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Award Number: DAMD17-00-1-0390

TITLE: A Novel Phosphatase Gene on 10q23, *MINPP*, in Hereditary and Sporadic Breast Cancer

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REPORT DATE: August 2003

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2003	Final (1 Aug 00-31 Jul 03)

4. TITLE AND SUBTITLE
A Novel Phosphatase Gene on 10q23, *MINPP*, in Hereditary and Sporadic Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0390

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

PTEN is a tumor suppressor gene on 10q23 and encodes a dual specificity phosphatase. One of the major substrates for PTEN is phosphotidylinositol (3,4,5) triphosphate in the PI3 kinase pathway. When PTEN is dysfunctional or absent, P-Akt is high and hence, anti-apoptotic. *PTEN* is a major susceptibility gene for Cowden syndrome (CS), a hereditary disorder with a high risk of breast and thyroid cancer, and appears to be involved in a broad range of tumors. In addition, germline *PTEN* mutations have been found in a developmental disorder, Bannayan-Riley-Ruvalcaba syndrome (BRR) as well. Previously not thought to be associated with cancer risk, BRR families and cases with germline *PTEN* mutations have recently been shown to be at risk for cancers and especially breast tumors. Between 10-80% (mean 60%) of CS families and 60% of BRR individuals have germline *PTEN* mutations. Families that do not have germline *PTEN* mutations are not inconsistent with linkage to the 10q22-23 region. Thus, genes with related function to *PTEN* in the 10q21-q25 region are good candidates genes for *PTEN* mutation negative CS, BRR and related sporadic tumors, eg, those of the breast and thyroid. *MINPP1* lies no more than 1 Mb upstream of *PTEN* and encodes an inositol polyphosphate phosphatase. While we have found no intragenic germline *MINPP1* mutation in CS and BRR, *MINPP1* has been found to be co-deleted with *PTEN* in one case of BRR with prominent GI features. *MINPP1* also appears to act as a low penetrance susceptibility gene for sporadic follicular thyroid neoplasia but not breast cancer. While no somatic intragenic *MINPP1* mutations were found in whole breast cancers, co-deletion of *PTEN* and *MINPP1* occur in ~30-40% of sporadic breast carcinomas. More interestingly, these codeletions have been shown to occur in the stroma. Therefore, *MINPP1* plays a comodulatory (non-traditional) role in hereditary and sporadic breast cancer.

14. SUBJECT TERMS
Human Cancer Genetics, Breast Cancer Genetics

15. NUMBER OF PAGES
49

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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Final Report

Proposal Title: A novel phosphatase gene on 10q23, *MINPP*, in hereditary and sporadic breast cancer (DAMD17-00-1-0390)
PI: Charis Eng, MD, PhD

INTRODUCTION

PTEN is a tumor suppressor gene on 10q23 and encodes a dual specificity phosphatase. One of the major substrates for PTEN is phosphotidylinositol (3,4,5) triphosphate in the PI3 kinase pathway. Downstream of this pathway lies Akt/PKB, a known cell survival factor. When PTEN is functional and abundant, Akt is hypophosphorylated and hence, pro-apoptotic. Conversely, when PTEN is dysfunctional or absent, P-Akt is high and hence, anti-apoptotic. *PTEN* is a major susceptibility gene for Cowden syndrome (CS), a hereditary disorder with a high risk of breast and thyroid cancer, and appears to be involved in a broad range of tumors. In addition, germline *PTEN*mutations have been found in a developmental disorder, Bannayan-Riley-Ruvalcaba syndrome (BRR) as well. This is an autosomal dominant disorder characterised by macrocephaly, lipomatosis, hemangiomatosis and speckled penis. Previously not thought to be associated with cancer risk, BRR families and cases with germline *PTEN* mutations have recently been shown to be at risk for cancers and especially breast tumors. Between 10-80% (mean 60%) of CS families and 60% of BRR individuals have germline *PTEN* mutations. Families that do not have germline *PTEN* mutations are not inconsistent with linkage to the 10q22-23 region. While breast cancer is a major component of CS and 30-50% of sporadic tumors carry hemizygous deletion in the 10q22-23 region, no or rare sporadic breast carcinomas have somatic intragenic *PTEN* mutations. A gene encoding a novel inositol polyphosphate phosphatase, *MINPP*, with overlapping function with PTEN, has been mapped to 10q23. We hypothesise that *MINPP* will be the susceptibility gene for the remainder of CS and BRR families and might likely be the major tumor suppressor gene on 10q23 which plays a role in the pathogenesis of sporadic breast carcinomas. We hope to explore whether *MINPP* is another CS and BRR susceptibility gene by looking for germline mutations in cases without germline *PTEN* mutations. We will also perform mutation and fine structure deletion analysis of *MINPP* in sporadic breast carcinomas. And finally, to prove that *MINPP* is a tumor suppressor and to begin to explore its relationship with PTEN in breast carcinogenesis, we will perform stable transfection experiments into two breast cancer lines with known genomic *PTEN* status (one PTEN wildtype and one PTEN null) as well as known PTEN protein and P-Akt levels. We will especially determine if MINPP is growth suppressive like PTEN, and determine if growth suppression is mediated by G1 arrest and/or apoptosis. Towards these ends, our specific aims were:

1. To determine if germline mutations of *MINPP* cause *PTEN* mutation negative CS, BRR and CS-like families.
2. To determine if somatic *MINPP* mutations and deletions are associated with sporadic breast carcinomas.

3. To determine if MINPP affects Akt activity and causes G1 arrest qand/or cell death in breast cancer cell lines.

BODY

Task 1: Mutation analysis of *MINPP* in germline *PTEN* mutation negative CS, BRR and CS-like Cases

Thirty unrelated CS probands, 35 unrelated BRR probands and 15 unrelated Proteus syndrome probands known not to harbor germline *PTEN* mutations by PCR-based DGGE and sequencing have thus far been ascertained for this particular task. CS and BRR were diagnosed stringently by the criteria of the International Cowden Consortium (1) and as documented previously(2), respectively. The criteria for the diagnosis of a CS-like individual or family is as previously described (3). Preliminary mutation analysis of all exons, exon-intron junctions and flanking intronic sequences of *MINPP1* have been performed on all these probands using a combination of PCR-based DGGE and direct sequencing. Amongst these 80 probands, no intragenic *MINPP* germline mutations have been found to date.

We then came up with an extended hypothesis that while germline intragenic mutations in *MINPP1* are going to be rare in *PTEN* mutation negative (by PCR-based strategies) CS/BRR/CS-like, large germline deletions and rearrangements involving *MINPP1* might occur. To test this hypothesis, a comprehensive approach to look for hemizygous mutations in the 10q22-q23 region encompassing *MINPP1* and *PTEN* was instituted. This included examining for possible hemizygosity of 10 microsatellite markers spanning this region as well as using real-time PCR to look for true germline hemizygosity. Microsatellite analysis and real-time PCR using probes only to exons 3 and 5 of *PTEN* have been completed for all 123 unrelated probands with classic CS and BRR. Interestingly, while no CS (of 95) probands were found to carry large deletions, 4 of 28 BRR probands were found to have deletions of the 10q22-q23 region (4) (Zhou and Eng, unpublished) [see attached reprint]. All 4 deletions include all or part of *PTEN*. Only 1 extended 5' to include *MINPP1*. This proband had prominent gastrointestinal hamartomatous polyposis.

In the last 2 years, another gene 5' of *MINPP1* called *BMPR1A* encoding a receptor for the bone morphogenetic proteins (BMPs) which belong to the TGFB superfamily was uncovered as a susceptibility gene for juvenile polyposis. We examined this gene in *PTEN* mutation negative CS and BRR and found 2 CS families with germline *BMPR1A* mutations (5) [see appendix] (Eng et al unpublished). These 2 CS families were particularly characterized by colonic features, which is relatively unusual for classic CS families. See attached reprint as well.

Task 2: Mutation and deletion analysis of *MINPP* in sporadic primary human breast carcinomas

To further understand the role of *MINPP* in sporadic counterparts of CS component cancers, we have accrued two series of sporadic tumors, primary adenocarcinomas of the

breast and primary follicular thyroid neoplasias. Currently, we have examined the 100 sporadic breast carcinomas for somatic *MINPP* mutations. To date, no obvious pathogenic intragenic mutations have been found although LOH in that region occurs with ~40% frequency (Matsumoto and Eng, unpublished).

In this series of breast cancers, we have also begun to look for somatic deletions in the *MINPP1* and *PTEN* regions using LOH analysis. However, working from the hypothesis that LOH can occur equally in the neoplastic epithelium as well as the surrounding stroma, we analyzed 50 sporadic invasive ductal carcinomas for LOH at markers D10S581, D10S579, D10S1765 and D10S541 for LOH in epithelium and stroma, which have been separated by standard laser capture microdissection. Of the informative samples, 42% had LOH of at least 2 D10 markers in the epithelium alone 30% stroma alone and 15% in both compartments (6) [see appendix] (Eng et al, unpublished). See attached reprint as well. Extrapolating from these preliminary data, we have performed somatic mutation analysis of *PTEN* and *MINPP1* in the epithelium and stroma of 50 sporadic invasive carcinomas of the breast. No intragenic *MINPP1* mutations were found in either the epithelium or stroma. Fifteen samples (30%) had somatic *PTEN* mutations in epithelium and/or stroma, with 6 mutations occuring in the stroma (7). As a control, the 8-exon *SDHB*, a mitochondrial complex II gene not known to be involved in epithelial carcinogenesis, was sequenced and no mutations were detected.

Task 3: Functional studies of *MINPP* in *PTEN*+/+ and PTEN null breast cancer cell lines

MINPP1 cDNA constructs have now been made in pCR2.1 and in the mammalian expression system pUHD10-3 which contains a tetracycline-suppressible (Tet-off) promoter, as previously described for *PTEN* expression constructs (8). Stable transfections in MCF-7 breast cancer lines (endogenous *PTEN* and *MINPP1* wildtype) and in BT-549 (PTEN null) did not produce any obvious growth suppressive effect. Following similar experiments as previously performed in our lab (8-12), no G1 cell cycle arrest or apoptosis was noted subsequent to ectopic expression of *MINPP1*. This likely corroborates the observations after targeted deletion of *mipp1* in a murine model by our collaborators Drs. Hongbo Chi and Paul Reynolds (13). What they observed after targeted disruption of *minpp1* was rectal prolapse and no neoplasia even after >3 years of follow-up. This might be due to the overlapping function of *MINPP1* and other polyinositol phosphatases.

KEY RESEARCH ACCOMPLISHMENTS

- Germline *MINPP1* deletions, with or without accompanying *PTEN* deletion, may account for an unknown subset of *PTEN* mutation negative (by PCR) CS and BRRS.

- *BMPR1A* might be a rare new susceptibility gene for *PTEN* and *MINPP* mutation negative CS and BRR, and might be associated with colonic phenotype.

- Somatic *MINPP1* alterations play some role in the genesis of sporadic follicular thyroid neoplasias.

- Germline *MINPP1* variation may be considered low penetrance alleles for predisposition to FTC.

- Germline *MINPP1* deletion as well as deletion of *PTEN* might be germane in rare BRR probands with prominent gastrointestinal polyposis.

- Somatic *MINPP1* deletion in the tumor stroma may play a role in invasiveness and progression in sporadic invasive adenocarcinomas of the breast.

REPORTABLE OUTCOMES

Dahia PLM, Gimm O, Chi H, Marsh DJ, Reynolds PR, **Eng C**. Absence of germline mutations in *MINPP1*, a phosphatase-encoding gene centromeric of *PTEN*, in patients with Cowden and Bannayan-Riley-Ruvalcaba syndrome without germline *PTEN* mutations. J Med Genet 2000; 37:715-7.

Gimm O, Chi H, Dahia PLM, Perren A, Hinze R, Komminoth P, Dralle H, Reynolds PR, **Eng C**. Somatic mutation and germline variants of *MINPP1*, a phosphatase gene located in proximity to *PTEN* on 10q23.3, in follicular thyroid carcinomas. J Clin Endocrinol Metab 2001; 86:1801-5.

Zhou XP, Woodford-Richens K, Lehtonen R, Kurose K, Aldred M, Hampel H, Launonen V, Virta S, Pilarski R, Salovaara R, Bodmer WF, Conrad BA, Dunlop M, Hodgson SV, Iwama T, Järvinen H, Kellokumpu I, Kim JC, Leggett B, Markie D, Mecklin J-P, Neale K, Phillips R, Piris J, Rozen P, Houlston R, Aaltonen LA, Tomlinson IPM, **Eng C**. Germline mutations in *BMPR1A/ALK3* cause a subset of juvenile polyposis syndrome and of Cowden and Bannayan-Riley-Ruvalcaba syndromes. Am J Hum Genet 2001; 69:704-11.

Kurose K, Hoshaw-Woodard S, Adeyinka A, Lemeshow S, Watson P, **Eng C**. Genetic model of multi-step breast carcinogenesis involving the epithelium and stroma: clues to tumour-microenvironment interactions. Hum Mol Genet 2001; 10:1907-13.

Kurose K, Gilley K, Matsumoto S, Watson PH, Zhou ZP, **Eng C**. Frequent somatic mutations in *PTEN* and *TP53* are mutually exclusive in the stroma of breast carcinomas. Nature Genet 2002; 32:355-7.

Zhou XP, Waite KA, Pilarski R, Hampel H, Fernandez MJ, Bos C, Dasouki M, Feldman GL, Matloff E, Ivanovich J, Greenberg LA, Patterson A, Pierpont ME, Russo D, Nassif NT, **Eng C**. Germline *PTEN* promoter mutations and deletions in Cowden/Bannayan-Riley-Ruvalcaba syndrome result in aberrant PTEN protein and dysregulation of the phosphoinositol-3-kinase/Akt pathway. Am J Hum Genet 2003; 73:404-11.

Elected to Membership, American Society of Clinical Investigation, Apr., 2001

Conferred the William C. and Joan E. Davis Professorship of Cancer Research by The Ohio State University, Oct., 2001.

Appointed Co-Director, Division of Human Genetics, Department of Internal Medicine, The Ohio State University, Sept., 2001.

Promoted to Professor of Medicine, Human Cancer Genetics and Molecular Genetics, The Ohio State University, July 1, 2002.

Novartis-Harry de Lozier Lecturership, Jan., 2002.

Stephanie Spielman Breast Cancer Research Award, James Cancer Hospital and Solove Research Institute, The Ohio State University, Jul, 2002.

Promoted to Director, Division of Human Genetics, Department of Internal Medicine, The Ohio State University, Oct., 2002.

Doris Duke Distinguished Clinical Scientist Award, Dec, 2002 (2002-07).

Conferred the Dorothy E. Klotz Endowed Chair in Cancer Research by The Ohio State University, Dec., 2002.

CONCLUSIONS

The third year of work exploring MINPP as an alternative phosphatase in playing a role in the etiology and pathogenesis of CS and sporadic tumors has suggested that *MINPP1* may not be a traditional high penetrance gene. These last 3 years of investigation has shown that traditional intragenic mutations in *MINPP1* do not occur in the germline of CS/BRR probands without germline intragenic *PTEN* mutations. Instead, it does participate as one of the genes deleted with *PTEN* in 1 of 4 deletion positive BRR cases with prominent gastrointestinal features. Similarly, *MINPP1* variants appear to confer low penetrance susceptibility to sporadic follicular thyroid neoplasias. Thus, in the germline, *MINPP1* appears to be a gene which plays a modulatory role rather than a traditional high penetrance role. Somatically, the observations mirror the germline observations. Intragenic somatic mutations are never seen in sporadic breast and thyroid neoplasias but it is often co-deleted with *PTEN* in especially breast carcinomas. The most interesting data are that these deletions not only occur in the neoplastic epithelium but also in the tumor stroma. Thus, *PTEN* deletion which often coexists with *MINPP1* deletion appears not only to add to the pathogenesis of breast cancers from the traditional epithelial cell origin but must also play an important role in the stroma perhaps contributing to invasiveness and metastases.

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APPENDIX

NIH-Style Biosketch

Six Reprints:

Dahia PLM, Gimm O, Chi H, Marsh DJ, Reynolds PR, **Eng C**. Absence of germline mutations in *MINPP1*, a phosphatase-encoding gene centromeric of *PTEN*, in patients with Cowden and Bannayan-Riley-Ruvalcaba syndrome without germline *PTEN* mutations. J Med Genet 2000; 37:715-7.

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Kurose K, Gilley K, Matsumoto S, Watson PH, Zhou ZP, **Eng C**. Frequent somatic mutations in *PTEN* and *TP53* are mutually exclusive in the stroma of breast carcinomas. Nature Genet 2002; 32:355-7.

Zhou XP, Waite KA, Pilarski R, Hampel H, Fernandez MJ, Bos C, Dasouki M, Feldman GL, Matloff E, Ivanovich J, Greenberg LA, Patterson A, Pierpont ME, Russo D, Nassif NT, **Eng C**. Germline *PTEN* promoter mutations and deletions in Cowden/Bannayan-Riley-Ruvalcaba syndrome result in aberrant PTEN protein and dysregulation of the phosphoinositol-3-kinase/Akt pathway. Am J Hum Genet 2003; 73:404-11.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel in the order listed for Form Page 2.
Follow the sample format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME	POSITION TITLE
ENG, Charis, MD, PhD	Professor of Medicine / Principal Investigator

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Chicago	BA	1978-82	Biological Sciences
University of Chicago	PhD	1982-86	Developmental Bio
University of Chicago	MD	1982-88	Medicine
University of Cambridge	Postdoc	1992-95	Human Cancer Genetics

A. Positions and Honors

1988-91 Residency in Internal Medicine, Beth Israel Hospital, Boston, MA

1991-94 Clinical Fellowship, Medical Oncology, Dana-Farber Cancer Institute, Boston, MA

1992-95 CRC Dana-Farber Fellowship in Human Cancer Genetics, University of Cambridge, UK

1992-95 Senior Registrar in Clinical Cancer Genetics, University of Cambridge Addenbrooke's Hospital, Cambridge, UK and Royal Marsden Hospital, London, UK

1994-95 Instructor in Medicine, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA

1995-98 Assistant Professor of Medicine, Dana-Farber Cancer Institute and Harvard Medical School, Boston

1995-98 Active Staff Physician, Adult Oncology, Dana-Farber Cancer Institute, Boston

1995-98 Associate Physician, Brigham and Women's Hospital, Boston

1998- North American Editor and Cancer Genetics Editor, *Journal of Medical Genetics*

1999-2001 Associate Professor (with tenure) of Medicine, The Ohio State University, Columbus

1999- Director, Clinical Cancer Genetics Program, James Cancer Hospital and Solove Research Institute, Comprehensive Cancer Center, Ohio State University, Columbus

1999- Member, Molecular Biology and Cancer Genetics Program, Comprehensive Cancer Center, The Ohio State University, Columbus

2001-02 Co-Director, Division of Human Genetics, Dept of Internal Medicine, The Ohio State University, Columbus

2001- William C. and Joan E. Davis Professor of Cancer Research, The Ohio State University, Columbus

2002- Dorothy E. Klotz Chair in Cancer Research, The Ohio State University, Columbus

2002- Professor (with tenure) of Medicine and Human Cancer Genetics, The Ohio State University, Columbus

2002- Director, Division of Human Genetics, Dept of Internal Medicine, The Ohio State University, Columbus

1982 Phi Beta Kappa

1982 Sigma Xi Associate Membership and Sigma Xi Prize

1987 Sigma Xi Promotion to Full Membership

1988 Alpha Omega Alpha

1999 American College of Physicians, Promotion to Fellowship

2001 American Society for Clinical Investigation, Elected Membership

2001-03 American Society for Clinical Oncology Advisory Committee on Genetic Testing for Cancer Susceptibility

B. Selected Peer Reviewed Publications (out of a total of 200 published and in press)

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C. Research Support

"Genetic analysis of the role of the microenvironment in epithelial tumor progression"
PI's: Charis Eng, MD, PhD, Gustavo Leone, PhD and Michael C. Ostrowski, PhD
Agency: V Foundation Jimmy V Golf Classic Research Award 3/1/01-2/28/04
The goal of this award is to provide seed moneys to gather preliminary data and make reagents related to tumor-microenvironmental interactions so that a group grant, eg PPG, may result from such work, as well as novel targets for therapy and prevention

"RET complex polymorphisms in Hirschsprung disease"
PI: Charis Eng, MD, PhD
Agency: National Institutes of Health R01 Research Project Grant 7/1/01-6/30/05
The goal of this project is to identify and characterise common low penetrance alleles within *RET* and the genes which encode its ligands and co-ligands in "sporadic" medullary thyroid carcinoma as well as sporadic Hirschsprung disease

"Genetics of epithelial-stromal interactions in hereditary breast cancer"
PI: Charis Eng, MD, PhD
Agency: Department of Defense Idea Award 3/25/02-4/25/05
The goal of this study is to determine if there are differences in somatic genetic alterations in the neoplastic breast epithelium and surrounding stroma in breast cancer from *BRCA1* and *BRCA2* tumors compared to sporadic counterparts.

"Genetic alterations in the epithelial and stromal compartments of prostate adenocarcinomas"
PI: Charis Eng, MD, PhD
Agency: Department of Defense Idea Award 12/01/01-11/30/05
The goal of this study is to build a genome-wide genetic model of step-wise, multistage carcinogenesis in the prostate involving the epithelium and stroma

"Genetics of *PTEN* in Cowden and related syndromes and familial cancer"
PI: Charis Eng, MD, PhD
Agency: American Cancer Society Research Scholar Grant 7/1/02-6/30/06
The goal of the project is to determine the individual-as-unit *PTEN* genotype-organ-specific phenotype risk of cancer in individuals with *PTEN* mutations, and to determine the risk and age of onset of each type of cancer.

"Genetic etiologies of esophageal Barrett's and cancer"

PI: Charis Eng, MD, PhD
Agency: National Cancer Institute R21 Pilot Grant 9/1/02-8/31/04
This is a proposal to accrue families with Barrett esophagus and adenocarcinoma of the esophagus to begin whole genome scans and use expression microarrays to search for the predisposing gene(s).

"Genetics of *PTEN* and molecular-based patient care"
PI: Charis Eng, MD, PhD
Agency: Doris Duke Charitable Foundation Distinguished Clinical Scientist Award 12/15/02-12/14/07
This is an award for translational research and mentorship activities on the platform of the comprehensive analysis of *PTEN* in cancer as a paradigm for clinical cancer genetics translational research
Overlap: None

of *LDLR* as FH causing, as they appear to have a modest effect on LDL receptor function.

Karen Heath is a PhD student sponsored by the John Pinto Foundation and financial support is from British Heart Foundation grants (RG95007 and RG93008).

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Absence of germline mutations in *MINPP1*, a phosphatase encoding gene centromeric of *PTEN*, in patients with Cowden and Bannayan-Riley-Ruvalcaba syndrome without germline *PTEN* mutations

EDITOR—Germline mutations in the dual specificity phosphatase gene *PTEN* (also known as *MMAC1* or *TEP1*) have been associated with susceptibility to two related hamartomatous disorders, Cowden syndrome (CS, MIM 158350) and Bannayan-Riley-Ruvalcaba syndrome (BRR, MIM 153480).[1 2] It has recently been established that PTEN functions as a 3-phosphatase towards phospholipid substrates in the phosphatidylinositol 3-kinase (PI-3 kinase) pathway.[3] Lack of PTEN results in the accumulation of phosphatidylinositol-(3,4,5)-P_3, which is required for activation of protein kinase B (PKB)/Akt, a downstream target of PI3-kinase and a known cell survival factor.[4–8]

While up to 81% of CS and approximately 60% of BRR cases have detectable *PTEN* germline mutations, no mutations in the coding region or exon-intron boundaries of *PTEN* have been found in the remaining affected subjects.[2 9–13] Informative *PTEN* mutation negative families have been shown to be linked to the 10q23 region, where *PTEN* lies,[2 14] although recently there has been a report of two CS families in which linkage to 10q23 has been excluded.[9] This has raised the possibility that either a regulatory region of the *PTEN* gene not included in previous studies, such as the promoter region, or another, closely located gene might be responsible for the CS and BRR cases in which no *PTEN* mutation has been found. The first alternative is unlikely to represent the majority of such cases, as no evidence of *PTEN* transcriptional silencing has been detected in the tissue of affected CS subjects in which no *PTEN* mutation was identified (Dahia and Eng, unpublished observations). Transcription levels of PTEN were found to be similar in affected and unaffected tissues of at least three unrelated CS patients and were equivalent to those of normal subjects. This suggests that methylation of the promoter or mutation within the promoter affecting transcription of *PTEN* does not occur in at least a subset of these *PTEN* mutation negative CS and BRR cases. To investigate the possibility that a closely mapped gene was the target of such mutations, we examined the coding region of a recently identified gene mapping to 10q23, next to D10S579, a marker estimated to lie no more than 1 Mb centromeric of *PTEN*.[15] The multiple inositol polyphosphate phosphatase, known as *MINPP1* or *MIPP*, has been cloned and shown to encode a conserved domain common to histidine phosphatases.[15 16] *MINPP1* codes for an approximately 52 kDa enzyme with the ability to remove the 3-phosphate from inositol phosphate substrates, such as Ins (1,3, 4,5)P_4, as well as other inositol moieties. It has been shown that human *MINPP1* has a wide tissue distribution pattern and its subcellular localisation appears to be targeted to the endoplasmic reticulum (ER).[15 16] While little is known about the human *MINPP1* function, its most well studied homologue, chick *HiPER1*, has a more restricted tissue distribution and appears to be critical to regulate the transition

Table 1 Primer sequences and annealing temperature used in PCRs of the MINPP1 gene

MINPP1 exon	*Forward sequence*	*Reverse sequence*	*Annealing temperature used for PCR*
1-A	MINPP1 5'UTRF CTCCACTGACCGTCCCGA	MINPP1-296R ATCTGTTTGACCGTGGGGTA	54
1-B	MINPP1-145F ACCAAGACTCGCTACGAGGA	MINPP1-556R GTGCTTGGAACTGGTGATGA	54
1-C	MINPP1-535F CTCATCACCAGTTCCAAGCA	MINPP1-I-1R: AGGACCGGGACAGCACAC	61
2	MINPP1-I-2F: CGGCTGTGCGGATTAGTAAG	MINPP1-I-2R: TCCTTATGTTTTCATTTTCACAGTTC	54
3	MINPP1-I-3F: TCCCCAAACTGAAGATGTCC	MINPP1-I-3R: AACCAAATGCAAACAAGCAA	54
4	MINPP1-I-4F: TCAGGGAATCTTGTTATATTTTTGAA	MINPP1-I-4R: TGGGTAGAGTGGAAGGTTCG	54
6*	MINPP1-1093F ATCCTCCAGTTTGGTCATGC or GTCTCAGCCAATTTCTTCTC	MINPP1-1464R TCATAGTTCATCAGATGTACTGTT	54

*In the chick *MINPP1* homologue, *HiPER1*, an extra exon, dubbed exon 5, and not seen in humans, precedes the final exon, named therefore exon 6.

of growth plate chondrocytes from proliferation to hypertrophy.[17] It is presumed that human *MINPP1* plays a role in differentiation and apoptosis, although details on the pathways involved in such signalling are as yet unknown. Thus, owing to its chromosomal location and to the fact that, like *PTEN*, it encodes a phosphatase with activity towards lipid substrates, we sought to investigate whether mutations in *MINPP1* would account for cases of CS and BRR without detectable *PTEN* mutations.

We obtained DNA from 36 subjects who met stringent criteria for the diagnosis of CS (n=14) and BRR (n=22) and in whom no *PTEN* mutation had been detected.[12 13] In at least one of the families, linkage data were compatible with linkage of the CS phenotype with the 10q23 region.[14] The rest of the cases were isolated or belonged to small families where linkage analysis was impossible. Informed consent was obtained from all subjects enrolled in this study, according to institutional Human Subjects Protection Committee protocols. All samples were screened for mutations in the coding region of *MINPP1* and most intron-exon boundaries of the gene by PCR based (primer sequences and PCR conditions in table 1) direct sequence analysis, as previously described.[18] No *MINPP1* mutations were found in germline DNA from any of the subjects examined in the present study. In particular, no mutations were found at the highly conserved histidine phosphatase motif, RHGxRxP, which defines members of the histidine acid phosphatase family. In addition, a second highly conserved site in this group of phosphatases comprising a histidine residue located at position 370 was found to be intact in all samples examined. This represents a proton donor site at the carboxy-terminal region of the protein which appears to be critical for full catalytic activity of this group of enzymes.[15 16] We identified five variations from the reference *MINPP1* sequence from the database in all samples, as well as in three normal controls (GenBank accession number AF046914). All of these sequence variants were identical to the reference *MINPP2* sequence (GenBank accession number AF084943). A sixth variant, c.444A→G, was noted in all our sequences which is in agreement with the *MINPP1* reference sequence, but at odds with that of *MINPP2*. It is likely, therefore, that these variations might represent errors in sequence entry on the database, rather than being associated with any particular phenotype, as they were identical in all samples, including the normal controls.

While described as independent hamartoma syndromes with shared clinical features until recently, it has been generally accepted that only CS bears a higher susceptibility to malignancies.[19 20] A broad analysis of genotype-phenotype data in the largest series of both CS and BRR recently undertaken in our laboratory has suggested that they might in fact represent distinct spectra of the same primary disorder.[13] These findings have clear implications for the follow up of affected subjects, in which systematic cancer surveillance is now recommended for both disorders, and not only for patients with CS.

In several human malignancies, such as breast, prostate, and thyroid cancer with loss of heterozygosity of 10q and in which no *PTEN* mutations have been found, it has been suggested that a region proximal to *PTEN* might be the main target in the tumorigenesis pathway.[18 21–24] It remains to be determined whether somatic abnormalities of *MINPP1* might be related to any of these sporadic tumours.

In conclusion, we have excluded an important candidate gene as the primary genetic abnormality underlying CS and BRR in subjects without identifiable *PTEN* mutation. It is possible that some degree of genetic heterogeneity exists, as suggested by a study that has excluded linkage to 10q23 in two *PTEN* mutation negative CS families.[9] The major genetic defect responsible for CS and BRR in cases without detectable *PTEN* mutation still remains to be established.

This work was partially supported by the US Army Breast Cancer Research Program (to CE) and P30CA16058 from the National Cancer Institute, Bethesda, MD (Ohio State University Comprehensive Cancer Center). PLMD is a Susan G Komen Breast Cancer Research Foundation Postdoctoral Research Fellow (to CE) and OG a Fellow of the DFG.

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J Med Genet 2000;37:717–719

Mosaicism in Alport syndrome and genetic counselling

EDITOR—Alport syndrome is characterised by a progressive glomerulonephritis with typical ultrastructural changes in the glomerular basement membrane. The most frequent, semidominant, X linked type is the result of a variety of mutations (either point mutations or intragenic deletions) of the *COL4A5* gene encoding the α5 chain of type IV collagen.[1]

During SSCP scanning of the *COL4A5* gene, a shift in a segment including exon 44 and flanking intronic sequences was found in a 19 year old proband showing typical ultrastructural changes of the glomerular basement membrane (III.3 in fig 1). Sequence analysis showed a G→C transversion in the 5' splice site of intron 44 (position 4271+1). The mutation introduced an *Alu*I restriction site which divided a 66 bp fragment into two fragments of 39 + 27 bp. All 18 family members were tested using this restriction assay and the mutation was found in the proband's affected brother, his cousin, his mother, and two maternal aunts. Surprisingly, the proband's grandmother was a normal homozygote. The proband's grandfather was dead, but true paternity of all daughters could be (indirectly) ascertained by polymorphic markers.[4]

In this family the mutation is associated with juvenile Alport syndrome in males, suggesting that the splicing defect results in a low level or absence of the protein, in agreement with our previous findings on genotype-phenotype correlations.[1] Interestingly, we noted considerable clinical variability among heterozygous females (n=4), ranging from ESRD at 27 years to absence of microscopic haematuria at 37 years.

Our data strongly suggest mosaicism in the germ cells of either grandparent. Mosaicism in germ cells may be the result of either a mutation in a germ cell that thereafter undergoes mitotic divisions (giving rise to mosaicism confined to germ cells), or an early postzygotic mutation before separation of the somatic/germ cells (giving rise to mosaicism in both the tissues and germline). In the latter case, the phenotype may or may not be expressed in the mosaic subjects, depending on the proportion of mutated cells in the relevant tissues. In order to verify mosaicism in somatic tissues of the living grandmother (I.1 in fig 1), we used Amplification Refractory Mutation System (ARMS-PCR), a tool able to detect known mutations even when present in a low fraction of template molecules.[5] The primer sense for exon 44[1] was used in combination with the following specific antisense primers: normal (5'-GGTATAACTATCTTCAGGAATAAGTCTTAC-3') and mutant (5'- GGTATAACTATCTTCAGGAATAAGTCT TAG-3'). We performed ARMS-PCR on DNA extracted from grandmaternal peripheral blood using progressively lower stringency by lowering the temperature or increasing the PCR cycle number or both, with the aim of reaching a condition where even the very few mutated molecules present in the blood sample would be amplified. This condition was never reached, as the grandmother's DNA always gave the same results as normal homozygous female controls (data not shown).

On analysis of Xq22 DNA polymorphisms, the three carrier females in the second generation were homozygous for one of the maternal haplotypes, which therefore must have been present in the dead grandfather as well, while the single non-carrier female and the unaffected male carried the other maternal haplotype. These data might suggest that the mutation was present in the grandmaternal gonads on

0021-972X/01/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 86, No. 4
Printed in U.S.A.

Somatic Mutation and Germline Variants of *MINPP1*, a Phosphatase Gene Located in Proximity to *PTEN* on 10q23.3, in Follicular Thyroid Carcinomas*

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ABSTRACT

Various genes have been identified to play a role in the pathogenesis of follicular thyroid tumors. Cowden syndrome is the only known familial syndrome with an increased risk of both follicular thyroid adenoma (FA) and carcinoma (FTC). Germline mutations in the tumor suppressor gene *PTEN*, which encodes a dual-specificity phosphatase, have been found in up to 80% of patients with Cowden syndrome suggesting a role of *PTEN* in the pathogenesis of follicular thyroid tumors. Although somatic intragenic mutations in *PTEN*, which maps to 10q23.3, are rarely found in follicular tumors, loss of heterozygosity (LOH) of markers within 10q22–24 occurs in about 25%. Recently, another phosphatase gene, *MINPP1*, has been localized to 10q23.3. MINPP1 has the ability to remove 3-phosphate from inositol phosphate substrates, a function that overlaps that of PTEN. Because of this overlapping function with *PTEN* and the physical location of *MINPP1* to a region with frequent LOH in follicular thyroid tumors, we considered it to be an excellent candidate gene that could contribute to the pathogenesis of follicular thyroid tumors. We analyzed DNA from tumor and corresponding normal tissue from 23 patients with FA and 15 patients with FTC for LOH and mutations at the *MINPP1* locus. LOH was identified in four malignant and three benign tumors. One of these FTCs with LOH was found to harbor a somatic c.122C > T or S41L mutation. We also found two germline sequence variants, c.809A > G (Q270R) and IVS3 + 34T > A. The c.809A > G variant was found in only one patient with FA but not in patients with FTC or normal controls. More interestingly, IVS3 + 34T > A was found in about 15% of FA cases and normal controls but not in patients with FTC. These results suggest a role for *MINPP1* in the pathogenesis of at least a subset of malignant follicular thyroid tumors, and *MINPP1* might act as a low penetrance predisposition allele for FTC. (*J Clin Endocrinol Metab* **86:** 1801–1805, 2001)

FOLLICULAR THYROID TUMORS are a common finding in iodine-deficient areas. By far, the most common tumors are benign follicular thyroid adenomas; only a minority of the tumors are carcinomas. Until today, it is unknown whether an adenoma-carcinoma sequence exists. Data supporting both theories exist (1–3).

The only known familial syndrome with an increased risk of both benign and malignant follicular thyroid tumors is Cowden syndrome (4). Germline mutations of *PTEN*, encoding a dual-specificity phosphatase, are found in up to 80% of patients with Cowden syndrome (5, 6), 60% of patients with Bannayan-Riley-Ruvalcaba syndrome (7), and an unknown proportion of patients with a Proteus-like syndrome (8). Although somatic intragenic *PTEN* mutations are found in only a minority of sporadic follicular thyroid carcinomas (9, 10), loss of heterozygosity (LOH) of markers within 10q23, especially including marker D10S579, has been found in up to 25% of either benign or malignant follicular tumors (9–11). In another study, fine structure deletion analysis of 10q22–24 demonstrated regions of loss that suggest that follicular adenomas and carcinomas develop along distinct parallel neoplastic pathways (11).

A new gene, *MINPP1* (multiple inositol polyphosphate phosphatase), has recently been localized to 10q23.3 in close proximity to marker D10S579 (12). *MINPP1*, also known as *MIPP*, has been shown to encode a conserved domain common to histidine phosphatases (12, 13). This 52-kDa enzyme has the ability to remove 3-phosphate from inositol phosphate substrates, such as Ins(1,3,4,5)P_4, a function that overlaps that of PTEN even though the sequence similarity of *PTEN* and *MINPP1* is only about 16%. MINPP1 is the only

Received December 27, 1999. Revision received November 29, 2000. Accepted December 2000.

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* This work was supported in part by the Department of Defense U.S. Army Breast Cancer Research Program (DAMD17-00-1-0390 to C.E.) and the National Cancer Institute (P30CA16058 to The Ohio State University Comprehensive Cancer Center).

† Recipient of a postdoctoral fellowship from the Susan G. Komen Breast Cancer Research Foundation (to C.E.).

enzyme known to hydrolyze the abundant metabolites inositol pentakisphosphate and inositol hexakisphosphate. Little is known about human *MINPP1*. It has been shown, however, to be expressed in a wide variety of tissues, including the human thyroid (Gimm, O., and C. Eng, unpublished data). Because of *MINPP1*'s overlapping function with *PTEN* and its physical location within a region of LOH for thyroid tumors, it is an excellent candidate gene that could contribute to thyroid tumorigenesis.

Here, we report the results of mutation analysis of *MINPP1* in benign and malignant follicular thyroid tumors from an iodine-deficient area. Our data might tentatively suggest a role of *MINPP1* in the tumorigenesis of at least a subset of malignant follicular thyroid tumors.

Materials and Methods

Patients and specimens

Paraffin blocks from 38 unselected benign (n = 23) and malignant (n = 15) follicular thyroid tumors were ascertained from Germany and Switzerland. Three malignant tumors were classified as Hürthle cell carcinoma. All samples were obtained with informed consent. In all 38 samples, tumor tissue and corresponding normal tissue (either normal thyroid tissue from a different block or from an area not in proximity to the tumor, or adjacent muscle tissue distant to the tumor site) were available for extraction of paired somatic and "germline" genomic DNA. DNA extraction following microdissection was performed using standard protocols (14).

Mutation analysis

PCR amplification using genomic DNA as template was carried out in 1× PCR buffer (Perkin-Elmer Corp., Norwalk, CT) containing 200 μM dNTP (Life Technologies, Inc., Gaithersburg, MD), 1 μM of each primer (see Table 1), 2.5U *Taq* polymerase (QIAGEN, Valencia, CA), 0.9 mM $MgCl_2$, 1× Q-buffer (QIAGEN), and 50–100 ng of tumor DNA template in a 50 μL volume. PCR conditions were 35 cycles of 1 min at 95 C, 1 min at 58 C, and 1 min at 72 C followed by 10 min at 72 C. All exons were at least divided into two (a and b) because of their large sizes. Exon 1 had to be divided into three fragments (a–c).

Mutation analysis for exon 2 (fragments 2a and 2b), exon 3 (fragments 3a and 3b), and exon 6 (fragments 6a and 6b) was performed with DGGE. Exon 1 and exon 4 of *MINPP1* are very GC rich and therefore less suitable for DGGE. Hence, mutation analysis for these two exons was performed using SSCP (fragments 1b, 1c, 4a, and 4b). No optimal SSCP condition could be found for the 5′ part of exon 1 (fragment 1a) and hence was subjected to direct semiautomated sequence analysis as previously described (5, 15, 16).

Before DGGE, 10 μl of the resulting PCR product were added to 1 μl of Ficoll-based loading buffer. This mixture was loaded onto 10% polyacrylamide gels carrying a 15–65% urea-formamide gradient and a 2–9% glycerol gradient in 0.5 × TAE. The amplicons were electrophoresed at 60 C and 105 V for 16 h. The fragments were visualized with ultraviolet transillumination after staining with ethidium bromide solution (15 μl in 500 ml dH_2O) for 30 min.

Before SSCP, 2 μl of the resulting PCR product were added to 3 μl of formamide buffer and then heated to 95 C for 10 min and subsequently cooled on dry ice. Immediately before SSCP, the samples were quickly thawed and then run through a 10% polyacrylamide/1 × TBE gel. Gels were run either at 100 V for 14 h at room temperature (fragments 1b, 4a, 4b) or at 150 V for 16 h at 4 C (fragment 1c). Subsequent silver staining was performed as previously described (17).

Although DGGE is 100% sensitive and specific in this and other laboratory's hands (18–20), SSCP is acknowledged not to have the same high sensitivity and specificity (21). Routine quality control for both SSCP and DGGE in our laboratory takes the form of subjecting a known positive and known negative to electrophoresis along with the test samples. Further, three random SSCP negative samples are subjected to direct sequence analysis.

If variant DGGE/SSCP banding patterns were observed, the remaining PCR aliquot was subjected to purification and semiautomated sequencing using the above primers and dye terminator technology (see above). If sequencing revealed a variant, the corresponding germline DNA was examined in the same manner to determine whether the sequence variant is somatic or germline.

The frequencies of these sequence variants in patients with follicular thyroid tumors and in a race-matched control group were determined

TABLE 1. *MINPP1* primer sequences and PCR-product sizes

Fragment	Primer name	Primer sequence (5′ to 3′)	PCR-product size
1-a	MINPP1-10F[a]	CCGTCCCGACGATGCTAC	241
	MINPP1-250R[a]	CCGTCCCGACGATGCTAC	
1-b	MINPP1-189F[a]	AACCCCGTGCTATTGTCG	300
	MINPP1-488R[a]	CTGTCGCATATCCTGCCG	
1-c	MINPP1-444F	ATGGACGGGCAGCTAGTAGA	251
	MINPP1-I-1R	AGGACCGGGACAGCACAC	
2-a	MINPP1-I-2F	CGGCTGTGCGGATTAGTAAG	292
	MINPP1-GC-2aR[b]	TCTGGTCCAGTTTTGAAGGC	
2-b	MINPP1-GC-2bF[b]	TTGGACCTCCAACAGTTAATGA	304
	MINPP1-I-2R	TCCTTATGTTTTCATTTTCACAGTTC	
3-a	MINPP1-I-3F	TCCCCAAACTGAAGATGTCC	244
	MINPP1-GC-3aR[b]	TCAAAAACATCACACCAAGGA	
3-b	MINPP1-GC-3bF[b]	CTGTTCATTTGACCTGGCAAT	185
	MINPP1-I-3R	AACCAAATGCAAACAAGCAA	
4-a	MINPP1-I-4F	TCAGGGAATCTTGTTATATTTTTGAA	177
	MINPP1-1071R[a]	CTGCTTTGTCCAAGTGCTGA	
4-b	MINPP1-1027F[a]	GCTGCACCTTGTTTCAGGAT	251
	MINPP1-I-4R	TGGGTAGAGTGGAAGGTTCG	
6-a[c]	MINPP1-E6F	GTCTCAGCCAATTTCTTCTC	294
	MINPP1-GC-6aR[b]	TTTCATTTAATAACATCTGCACTCG	
6-b[c]	MINPP1-GC-6bF[b]	CACTGTGAAAATGCTAAGACTCC	298
	MINPP1-1538R[a]	GCATGTAATCACTCATTGCAGA	

[a] The number refers to the number of the position of the 5′ end of the primer in the sequence available under accession number AF084943; it is not equal to the nucleotide number within the translated coding region.

[b] These primers have in addition a GC-rich clamp (5′-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3′) on their 5′ end in order to perform DGGE mutation analysis.

[c] In the chick *MINPP1* homolog, *HiPER1*, an extra exon, dubbed exon 5, and not seen in humans, precedes the final exon, named therefore exon 6.

using peripheral blood leukocyte DNA. This race-matched control group consisted of patients who were admitted to the Department of General Surgery, Halle, Germany, for nonthyroid-related diseases. Informed consent was given in all cases.

LOH analysis

For every germline-tumor pair, PCR reactions were carried out using 0.6 μM each of forward and reverse primer in 1× PCR buffer (QIAGEN), 4.5 mM $MgCl_2$ (QIAGEN), 1× Q-buffer (QIAGEN), 2.5 U HotStarTaq polymerase (QIAGEN), and 200 μM dNTP (Life Technologies, Inc.) in a final volume of 50 μL. Reactions were subjected to 35 cycles of 94 C for 1 min, 55–60 C for 1 min and 72 C for 1 min followed by 10 min at 72 C. LOH analysis for each germline-tumor pair was performed as previously described using markers flanking *MINPP1*, D10S541 (telomeric), D10S2491 (telomeric) (5, 22), and D10S1686 (centromeric) as well as the marker D10S579 that lies in close proximity to *MINPP1* (12). All forward primers were 5′-labeled with either HEX or 6-FAM fluorescent dye (Research Genetics, Inc., Huntsville, AL).

Statistical analysis

Differences in allele frequencies were calculated using the standard Chi-square test. A P value less than 0.05 was considered significant.

Results

Mutation analysis of all 5 exons of *MINPP1* from 38 follicular thyroid tumors revealed variants in 3 exons (fragments 1a, 2b, and 3b). Sequencing revealed one sequence variant each (Table 2). Corresponding germline DNA was examined for the presence of each of these variants.

We detected a sequence variant in one carcinoma, c.122C > T (S41L), in the 5′ end of exon 1 (fragment 1a) (Fig. 1A). This variant was absent in the corresponding germline (DNA from muscle) (Fig. 1B) and most likely represents a somatic missense mutation. Repeat PCR and sequencing confirmed the variant and excluded PCR errors. Thus, somatic S41L was found in 1 out of 15 carcinomas (7%).

The variants in exon 2 (fragment 2b), c.809A > G (Q270R) (Fig. 1, C and D) and fragment 3b, IVS3 + 34T > A (Fig. 1, F and G) were also present in the germline (data not shown). The heterozygous c.809A > G variant was seen in a patient with follicular thyroid adenoma and was never seen in patients with follicular thyroid carcinoma or in a race-matched control group (Fig. 1E and Table 2a).

The germline IVS3 + 34T > A variant was underrepresented in cases with follicular thyroid carcinoma (0%), compared with those with adenoma (15%; $P < 0.03$, Table 2b) or normal controls (14%; $P < 0.04$, Table 2b).

LOH analysis within 10q22–24 was performed for all tumor-germline pairs. We found LOH in seven follicular tumors, four carcinomas (27%), and three adenomas (13%). None of the seven follicular adenomas with IVS3 + 34T > A had LOH. Interestingly, the one carcinoma harboring the somatic mutation S41L showed LOH at D10S579, and the flanking markers D10S2491 and D10S1686 were not informative.

TABLE 2. Allele frequency of *MINPP1* polymorphic sequence variants in patients with follicular thyroid adenoma and follicular thyroid carcinoma and race-matched controls

Codon	Nucleotide (amino acid)	(a) Exon 2 FA	FTC	Controls
270	c.809G (Arg)	1	0	0
270	c.809A (Gln)	45	30	78

FA *vs.* FTC, P = n.s.; FA vs. controls, P = n.s.

Codon	Nucleotide (amino acid)	(b) Exon 3 FA	FTC	Controls
N/A	IVS3+34A (N/A)	7	0	9
N/A	IVS3+34T (N/A)	39	30	57

FTC *vs.* FA, $P < 0.03$; FTC *vs.* controls, $P < 0.04$. n.s., not significant; N/A, not applicable; FA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma.

Discussion

In the present study, we detected a somatic S41L mutation in *MINPP1* together with loss of the corresponding wild-type allele in one follicular thyroid carcinoma. We also found two previously unreported germline sequence variants in *MINPP1*; one, an intronic variant, is underrepresented in cases with follicular thyroid carcinomas, compared with those with follicular thyroid adenomas or normal controls.

The somatic mutation c.122C > T in tumor DNA from one patient with follicular thyroid carcinoma changes serine, a neutral and polar amino acid, at position 41, to leucine, which is also neutral but hydrophobic. This region is highly conserved among several species (human, rat, mouse) (12, 13). Hence, one can speculate that this polar for hydrophobic amino acid substitution changes the structure of *MINPP1*. Postulating that *MINPP1* might act as a tumor suppressor, its functional activity might subsequently be lost or at least decreased. However, functional analysis would be necessary to confirm this premature hypothesis. Nonetheless, loss of the corresponding wild-type allele in this sample lends credence that the somatic S41L mutation is pathogenic and both *MINPP1* alleles inactivated.

The finding of a rare germline sequence variant in one patient with follicular thyroid adenoma is intriguing. This variant was neither observed in 78 control alleles nor found in 36 patients with Cowden syndrome or Bannayan-Riley-Ruvalcaba syndrome (23). One may speculate that this variant, which leads to substitution of a neutral and polar amino acid for a basic amino acid, affects the function of *MINPP1*. Whether this hypothetical change of *MINPP1* function plays a role in the pathogenesis of follicular thyroid adenomas must remain unresolved at this point. Histological appearance did not show any unusual features. Also, there was no family history of follicular thyroid adenomas, but no germline DNA was available from any relative.

The absence of the relatively frequent intronic polymorphic sequence variant IVS3 + 34T > A in follicular thyroid carcinoma patients is intriguing. Even though our numbers are small, at least one or two follicular thyroid carcinomas harboring this sequence variant should have been detected: power calculations reveal that if only 10% of 30 alleles have this variant, our power to detect this in at least one case would exceed 0.92. We also screened 30 patients with breast cancer for variation in *MINPP1* and found about the same frequency (12%) of this polymorphism as found in patients with FA (15%) and controls (14%) (Gimm, O., and C. Eng, unpublished data). Of note, this intronic polymorphism lies on the border of a poly-T/poly-A/poly-T tract. There is some

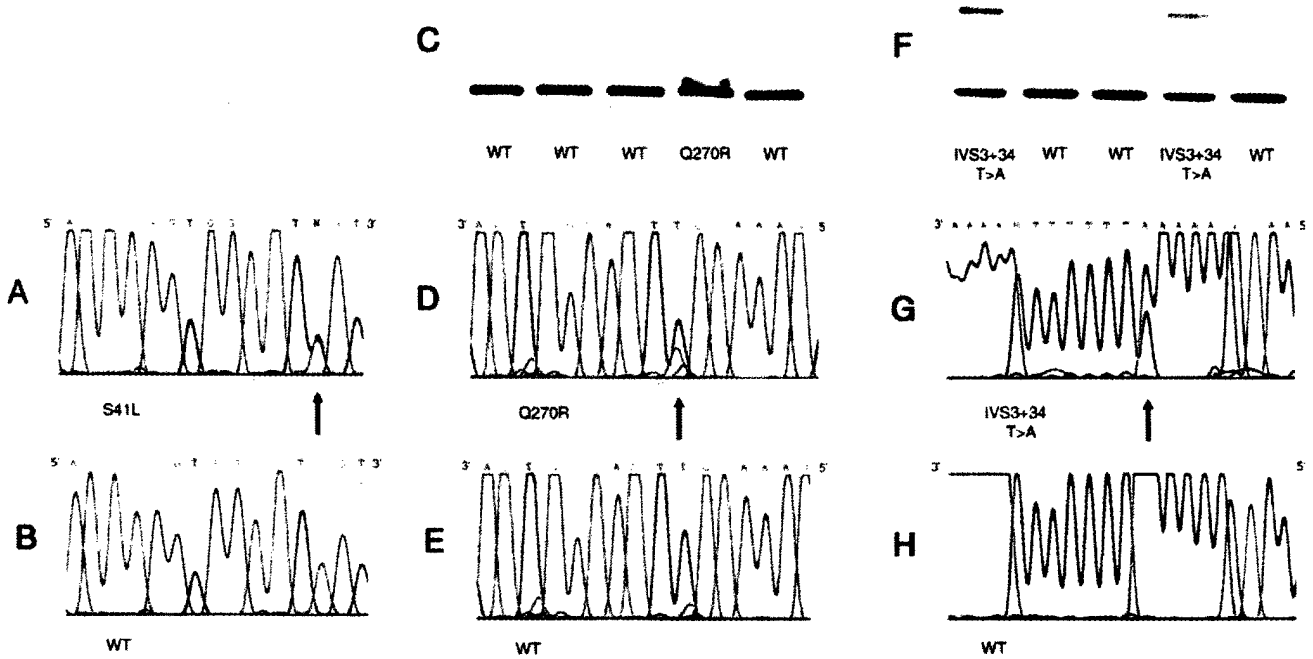

FIG. 1. Sequence variants in *MINPP1* identified by DGGE and/or sequencing. A, Sequence variant c.122C > T (S41L) in one follicular thyroid carcinoma. B, Absence of the sequence variant in the corresponding germline. C, DGGE reveals a variant in exon 2 (fragment 2b) in one follicular adenoma. D, Sequencing identifies the variant c.809A > G (Q270R). E, Example for absence of the variant c.809A > G (Q270R) in blood from one control patient. F, DGGE reveals a variant in exon 3 (fragment 3b) in follicular adenomas. G, Sequencing identifies the variant IVS3 + 34T > A. H, Example for absence of the variant c.809A > G (Q270R) in blood from one control patient. Note: Because the forward primers for the fragments 2b and 3b contain the GC-clamp, the reverse primers have been used for sequencing. Hence, the sequences in D, E, G, and H are reverse.

evidence that poly-N tracts may play important roles in RNA splicing and processing (24, 25). Recently, it has become more evident that development of a cancer can result from an interplay of either a few "high penetrance" mutations in key genes or from several, or many, sequence variants presently of unknown significance. For example, overrepresentation of a rare sequence variant of *RET* has been observed in patients with sporadic medullary thyroid carcinoma (26). Similar observations have been made for polymorphic sequence variants of *RET* in patients with HSCR (27, 28), *Cul2* in sporadic pheochromocytomas (29), and *PPARgamma* in isolated glioblastoma multiforme cases (30). However, the precise mechanism to explain how the intronic sequence variant could "protect" or at least lower the chance of developing follicular thyroid carcinoma is unknown and open to speculation. Possibly, this intronic change affects a splice-donor or splice-acceptor site or enhances a cryptic splice site, which would subsequently lead to a different protein. Unfortunately, complementary DNA was not available to test this hypothesis. The absence of this polymorphism in patients with follicular thyroid carcinoma may also support the hypothesis that follicular thyroid adenomas and carcinomas do not adhere to an adenoma-carcinoma sequence. Fine-structure deletion mapping of 10q22–24 also suggests that sporadic follicular thyroid adenomas and follicular thyroid carcinomas develop along distinct neoplastic pathways (11).

In conclusion, our observations suggest a role for *MINPP1* in the tumorigenesis of malignant follicular thyroid carcinoma. Although it may infrequently contribute to follicular carcinogenesis via the traditional pathway of somatic high penetrance, two-hit (31) mutations, this gene seems to harbor a variant that could act as a common low penetrance susceptibility allele for follicular thyroid carcinoma. Further, the DGGE and SSCP conditions reported here together with the knowledge of the frequency of various sequence variants may help in future mutation analyses of DNA from other cancers with LOH in the 10q23 region in which *PTEN* does not seem to play a major role, such as head and neck carcinomas, lung cancer, and melanomas (32).

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Germline Mutations in *BMPR1A/ALK3* Cause a Subset of Cases of Juvenile Polyposis Syndrome and of Cowden and Bannayan-Riley-Ruvalcaba Syndromes*

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Juvenile polyposis syndrome (JPS) is an inherited hamartomatous-polyposis syndrome with a risk for colon cancer. JPS is a clinical diagnosis by exclusion, and, before susceptibility genes were identified, JPS could easily be confused with other inherited hamartoma syndromes, such as Bannayan-Riley-Ruvalcaba syndrome (BRRS) and Cowden syndrome (CS). Germline mutations of *MADH4* (*SMAD4*) have been described in a variable number of probands with JPS. A series of familial and isolated European probands without *MADH4* mutations were analyzed for germline mutations in *BMPR1A*, a member of the transforming growth-factor β–receptor superfamily, upstream from the SMAD pathway. Overall, 10 (38%) probands were found to have germline *BMPR1A* mutations, 8 of which resulted in truncated receptors and 2 of which resulted in missense alterations (C124R and C376Y). Almost all available component tumors from mutation-positive cases showed loss of heterozygosity (LOH) in the *BMPR1A* region, whereas those from mutation-negative cases did not. One proband with CS/CS-like phenotype was also found to have a germline *BMPR1A* missense mutation (A338D). Thus, germline *BMPR1A* mutations cause a significant proportion of cases of JPS and might define a small subset of cases of CS/BRRS with specific colonic phenotype.

Introduction

The major hamartomatous-polyposis syndromes comprise juvenile polyposis syndrome (JPS [MIM 174900]), Peutz-Jeghers syndrome (PJS [MIM 175200]), Cowden syndrome (CS [MIM 158350]), and Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480]). Whereas CS and a subset of BRRS are allelic (Marsh et al. 1997*a*, 1999), current evidence suggests that CS and BRRS are genetically distinct from JPS and PJS (for reviews, see Eng and Ji 1998; Eng and Parsons 2001). PJS is an autosomal dominant disorder characterized by perioral pigmented spots, hamartomatous polyposis, and a risk for colon and breast cancers (Boardman et al. 1998; for review, see Eng et al. 2001). Germline mutations of the nuclear serine-threonine–kinase gene *LKB1/STK11* cause most cases of PJS (Hemminki et al. 1998; Jenne et al. 1998). CS is a poorly recognized autosomal dominant cancer syndrome characterized by multiple ha-

Received July 10, 2001; accepted for publication August 8, 2001; electronically published August 30, 2001.

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* To be presented, in part, as a slide presentation at the annual meeting of The American Society of Human Genetics, in San Diego, on October 14, 2001.

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0002-9297/2001/6904-0005$02.00

Table 1

Clinical Features Present in Probands and Families with JPS

	Cancer		Anomalies		
Proband or Family	Colon	Other	Cardiac	Head/Facial	Other
BMPR1A-mutation positive:					
16[a]	Yes	No	No	No	No
18[a]	Yes	Esophageal	No	No	No
10[a]	Yes	No	No	No	No
19[a]	Yes	Unknown	No	No	No
FT[a]	Yes	No	No	Hypertelorism, macrocephaly	No
SM316	No	No	No	Hypertelorism, macrocephaly	No
VR	Unknown	No	No	No	Porphyria, malrotation of gut
1469	Unknown	Unknown	Unknown	No	No
JP7/19[a]	Yes	No	Ventricular septal defect, unspecified defects	No	No
JP8/1	Yes	Adrenal hamartoma, Wilms tumor	Ebstein anomaly	No	No
BMPR1A-mutation negative:					
MD[a]	Yes	No	No	Hypertelorism, macrocephaly	Telangiectasia
5[a]	Yes	Stomach	No	No	No
6[a]	Yes	No	No	Macrocephaly	No
1[a]	Yes	Small bowel	No	No	No
14[a]	Yes	No	No	No	No
22[a]	No	No	No	Macrocephaly? (diameter 56 cm [female])	Pigment naevi on trunk
CV	Unknown	No	Unknown	No	No
KS[a]	Unknown	No	No	No	No
YC[a]	Yes	No	No	No	No
SM106	No	No	Ventricular septal defect	Hypertelorism	No
12[a]	Yes	Small bowel	Aortic regurgitation	Subarachnoid hemorrhage	No
DM[a]	Yes	Stomach	Unknown	No	No
WN[a]	Yes	No	Unknown	Unknown	Unknown
JP1/1	Yes	Melanoma	No	No	Osler disease, epilepsy, empty sellar syndrome
JP2/13[a]	Yes	No	Unknown	Unknown	Unknown

Note.—By definition, all probands or families have at least one member with juvenile polyps.

[a] Familial.

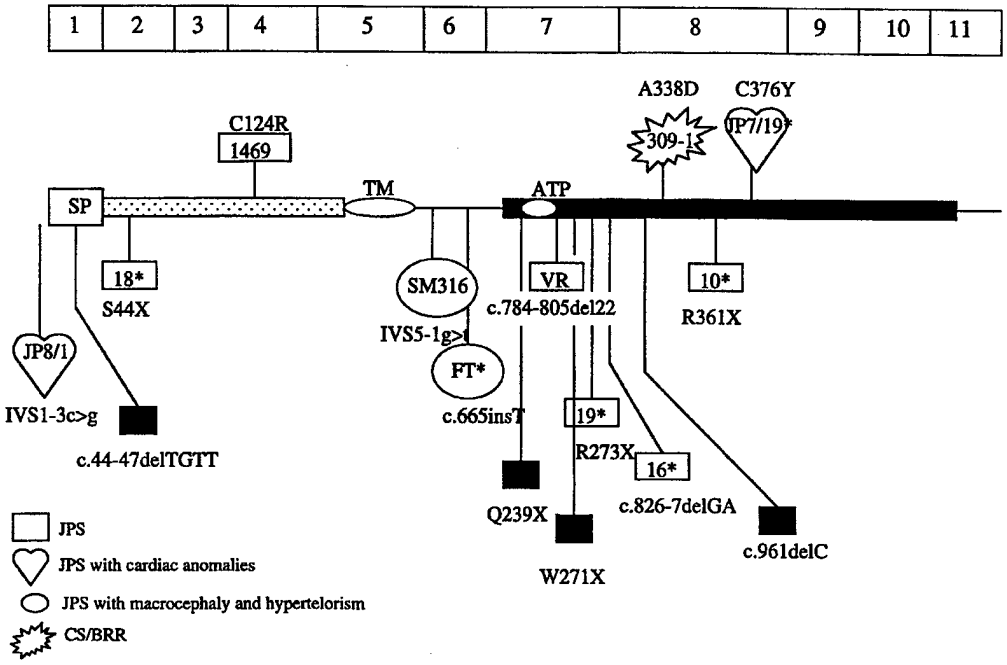

Figure 1 Spectrum of germline *BMPR1A* mutations in 14 probands with JPS and in 1 family with CS/BRRS. The exons are depicted by the numbered boxes at the top, and the domains of the receptor are depicted below. Signal peptide (SP), transmembrane domain (TM), ATP-binding domain (ATP), extracellular domain (*dotted bar*), and kinase domain (*black bar*) are shown. Black squares represent the four families' mutations published by Howe et al. (2001).

martomas and by a high risk for breast, thyroid, and endometrial cancers (Eng 2000). Although gastrointestinal hamartomatous polyposis can be documented if systematically searched for (Weber et al. 1998), the polyps are rarely symptomatic in CS, in contrast to the other three syndromes. BRRS is a congenital disorder characterized by macrocephaly, lipomatosis, thyroid problems, and pigmented macules on the glans penis in males (Gorlin et al. 1992); in BRRS, gastrointestinal hamartomatous polyposis can be quite prominent and symptomatic (Tsuchiya et al. 1998). Germline mutations in the tumor-suppressor gene *PTEN* cause 80% of cases of classic CS and 60% of cases of BRRS (Marsh et al. 1998*b*, 1999). There is little, if any, linkage evidence of genetic heterogeneity in CS (Nelen et al. 1996). The extent of genetic heterogeneity in BRRS is unknown. Clinical diagnosis of JPS is by exclusion, and JPS is characterized by gastrointestinal hamartomatous polyposis and by a risk for gastrointestinal cancers (for review, see Eng et al. 2001). Germline mutations in *MADH4* (*SMAD4*) have been described in a proportion of cases of JPS (Howe et al. 1998). From a nonsystematic survey of North American probands with JPS, it was estimated that ~35–60% of cases of JPS would harbor germline *MADH4* mutations (Howe et al. 1998); however, 3%–28% (weighted average 15%) of cases of JPS originating mainly from Europe have been found to carry *MADH4* mutations (Houlston et al. 1998; Friedl et al. 1999; Roth et al. 1999; Woodford-Richens et al. 2000*a*, and in press). Thus far, genes encoding several other SMADs have not been found to be associated with JPS (Bevan et al. 1999; Roth et al. 1999). Recently, germline truncating mutations in *BMPR1A/ALK3/SKR5* were described in four of four families segregating JPS (Howe et al. 2001). *BMPR1A*, on 10q21-q22, encodes a bone morphogenic-protein–receptor serine-threonine kinase that belongs to the transforming growth-factor β (TGFB)–receptor SMAD superfamily (for reviews, see Massagué 2000; Eng 2001). Members of the TGFB-receptor superfamily can homo-oligomerize or hetero-oligomerize.

We have examined *BMPR1A* for germline mutations, in a cohort of familial and sporadic cases of JPS, with the hypotheses that this mainly European cohort with a relatively low *MADH4*-mutation frequency would have a high frequency of *BMPR1A* mutations with a distinct mutational spectrum. Furthermore, because of the location of this gene in proximity to *PTEN* (Dahia et al. 2000) and, perhaps, because of its function, it also became a good candidate gene for susceptibility in *PTEN*-mutation–negative cases of CS and of BRRS.

Families, Material, and Methods

Families

Eighteen unrelated families with JPS and seven isolated cases of JPS were ascertained by clinical criteria described elsewhere (Marsh et al. 1997*b*) and were already known not to carry germline *MADH4* mutations. Although all families and individuals met the diagnostic criteria for JPS, some affected individuals had developed other tumors (table 1)—predominantly, colorectal adenomas and/or cancer—as is common in this condition (Woodford-Richens et al. 2000*a*). Twenty-one probands with CS/BRRS or CS/BRRS-like phenotype without germline *PTEN* mutations were ascertained by the revised operational diagnostic criteria of the International Cowden Consortium (Eng 2000) and by criteria described elsewhere (Marsh et al. 1998*a*, 1999). Probands and families with CS/BRRS-like phenotype have component features of CS/BRRS but do not meet the operational diagnostic criteria set forth by the International Cowden Consortium. All specimens were collected and analyzed, after informed consent was obtained, under protocols approved by each institution's Human Subjects Protection Committees.

Mutation Analysis

Genomic DNA was extracted from peripheral leukocytes, by standard protocols (Mathew et al. 1987). As template, 20–100 ng of DNA was used for 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, after a hot start) of PCR amplification of each of the 11 coding exons (thus, ALK3E3 corresponds to exon 1, etc.) and flanking intronic regions of *BMPR1A*, by use of the following primers: ALKE3F (5′-TCCAAAATTCAGTTGTATTCC-3′), ALKE3R (5′-CACATACATTACTAAAATGAACACTG-3′), ALKE4F (5′-GTCACGAAACAATGAGCTTT-3′), ALKE4R (5′-TTAAGAAGGGCTGCATAAAA-3′), ALKE5F (5′-CATTCAGACTCAAATTTCGTT-3′), ALKE5R (5′-TCTCATGGGTCCCAAATTA-3′), ALKE6F (5′-CCAAACCATTTCTAATTTTATCA-3′), ALKE6R (5′-CATGCTCCGACTTTTCTC-3′), ALKE7F (5′-CCAGGCTACCTAGAATTGAA-3′), ALKE7R (5′-AACAGCGGTTGACATCTAAT-3′), ALKE8F (5′-CCTCAAGGTTTTTCTTAGGG-3′), ALKE8R (5′-TCAACACACCATTCATGTCT-3′), ALKE9F (5′-TCATCAAGAGCTCAAACCTT-3′), ALKE9R (5′-ACCTCACTAGCCTTGTCAAA-3′), ALKE10F (5′-CCCTAGCCTATCTCTGATGA-3′), ALKE10R (5′-AACAGTGGGGCAAAGAAC-3′), ALKE11F (5′-TATTTTATTTTTGGCCCTCA-3′), ALKE11R (5′-TGATGAGTAAATCAACATAATCAG-3′), ALKE12F (5′-ATTTTTGTGCCCATGTTTT-3′), ALKE12R (5′-AATCACTTCTTCAGGGGACT-3′), ALKE13F (5′-ACTCAGTCCCCTGAAGAAGT-3′), and ALKE13R (5′-CTAGAGTTTCTCCTCCGATG-3′). The amplicons were gel- and column-purified and then were subjected to semiautomated PCR-based sequence analysis by an ABI-377a or a Perkin-Elmer 3700, as described elsewhere (Mutter et al. 2000).

Loss of Heterozygosity (LOH) Analysis

Available component tumors from *BMPR1A*-mutation–positive and *BMPR1A*-mutation–negative cases of JPS were subjected to LOH analysis with markers ALK3ca, ALK3ggaa, and D10S573, by techniques described elsewhere (Marsh et al. 1998*c*; Woodford-Richens et al. 2000*b*). Two component tumors from proband JP8/1 (table 1) was analyzed by sequencing of the amplicon containing the germline mutation, to examine allelic contribution.

Reverse-Transcriptase PCR (RT-PCR) Analysis

To assess the putative splice-site mutation in proband JP8/1, RNA was extracted from her component tumors, a Wilms tumor (table 1), and a colon carcinoma, and cDNA was synthesized. RT-PCR was performed using the primers 5′-GCATAGGTCAAAGCTGTTTGG-3′ and 5′-GCAAGGTATCCTCTGGTGCT-3′, with Ampli*Taq* Gold (Perkin-Elmer) at and annealing temperature of 60°C. Amplicons were fractionated through 2% agarose, were stained with ethidium bromide, and then were visualized with UV trans-illumination. Any aberrant bands noted on the gel were cut out of low-melting-point agarose, were gel- and column-purified, and then were subjected to sequence analysis.

Results

All 11 coding exons, splice junctions, and flanking intronic regions of *BMPR1A* were examined in 18 unrelated *MADH4*-mutation–negative families with JPS and in seven unrelated *MADH4*-mutation–negative individuals with isolated JPS. All available polyps from these cases showed no loss of SMAD4 expression. Overall, of 25 unrelated probands with JPS, 10 (40%) were found to have germline *BMPR1A* mutations (fig. 1): 6 (33%) of the 18 familial cases and 4 (57%) of the 7 isolated cases had germline mutations. In the mutation-positive familial cases in which samples from family members were available, the respective mutations were shown to segregate with affected status (data not shown)

Of the 10 germline *BMPR1A* mutations found in probands with JPS, all except 2 were nonsense, frameshift, or splice-site mutations predicted to result in truncated receptors (fig. 1). The missense mutations found in cases of JPS were examined in cohorts of 50 race-matched normal controls. None of the 100 normal control chromosomes were found to carry these missense mutations; furthermore, in the familial cases of JPS with C376Y, this mutation was found to segregate with dis-

ease. Loss of the wild-type allele in three component tumors—all of which were villous adenomas and two of which also had adenocarcinomatous components—from an affected family member was also demonstrated (fig. 2). The splice-site mutation IVS1-3c→g was shown to result in skipping of exon 1, and the component tumor (a Wilms tumor; table 1) from the proband had loss of the wild-type allele. A colorectal carcinoma from the proband with the IVS1 splice mutation did not show LOH. Thus, of five component tumors from *BMPR1A*-mutation–positive individuals, four were found to have loss of the wild-type allele. In contrast, 24 component tumors from 13 familial and isolated cases without germline *BMPR1A* mutations showed no LOH in that region (data not shown).

Although limited because of small sample size, genotype-phenotype associations were examined, especially those with respect to cardiac anomalies or to head/facial features (table 1 and fig. 1). Both among the 10 *BMPR1A*-mutation–positive families and among the 15 *BMPR1A*-mutation–negative families, 2 had cardiac anomalies; because of the limited size of sample, these were not considered statistically different. Similarly, there appeared to be no difference between the numbers of mutation-positive and mutation-negative families and individuals with macrocephaly or hypertelorism. Although there were only two probands/families with mutations as well as with the clinical features of hypertelorism and macrocephaly, both of these mutations—IVS5-1g→t (SM316) and c.665insT (FT)—occurred in the juxtatransmembrane domain (fig. 1).

Of 21 unrelated probands with CS/BRRS, without germline *PTEN* mutations, 1 was found to have a germline missense mutation, A338D, in exon 8 of *BMPR1A*. This missense alteration was not observed among 172 race-matched, geographically matched control chromosomes. Interestingly, the proband had only colonic polyposis, which comprised hamartomatous and adenomatous polyps and began at the age of 16 years, and lipomas. Her family history, however, comprised individuals with breast cancer, with renal-cell carcinoma, with brain tumor(s), and with melanoma. Taken together, these features constitute the minimum criteria (i.e., one major and three minor) for the diagnosis of CS (Eng 2000). It is acknowledged that the diagnosis of CS in this family barely met the minimum International Cowden Consortium diagnostic criteria, and some clinicians might consider this family to have a CS-like phenotype. None of the other probands with CS/BRRS or CS/BRRS-like phenotype were found to have *BMPR1A* mutations.

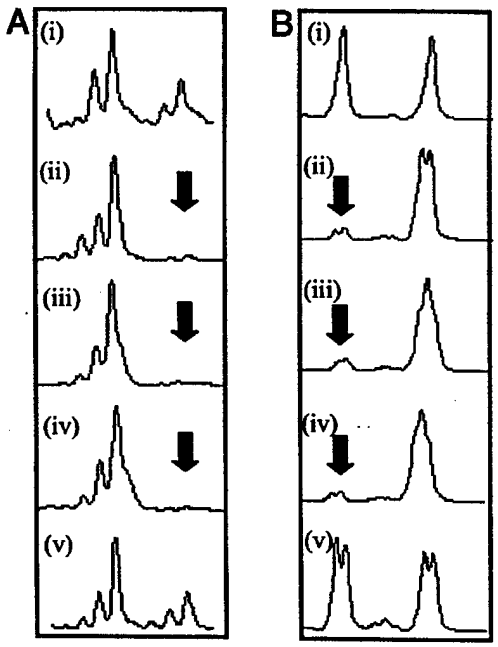

Figure 2 LOH analysis with microsatellite markers alk3ca (*A*) and alk3ggaa (*B*), which lie in proximity to *BMPR1A* (see text), and genomic DNA templates from family JP7/19, whose members harbor a germline missense mutation, generated from peripheral blood leukocytes (*i*), from villous adenoma (*ii*), from two villous adenomas with adenocarcinomatous components (*iii* and *iv*), and from normal tissue originating from the same archival section as one of the villous adenomas with adenocarcinoma (*v*).

Discussion

In this cohort of familial and isolated cases of JPS who are *MADH4*-mutation–negative and who originate mainly from Europe, 40% have been found to harbor germline *BMPR1A/ALK3* mutations. Thus, among European cases of JPS, *MADH4* mutations account for ≤28% of cases and *BMPR1A* mutations account for 40%. No systematic survey of cases of JPS originating in the United States has been performed yet, and it thus is unknown what proportion cases of JPS is due to *BMPR1A* mutations. Nonetheless, at least one other JPS-susceptibility gene should exist.

Overall, to date, 14 different germline *BMPR1A* mutations have been described in probands with JPS—10 in patients from this study and the 4 in U.S. kindreds described elsewhere (Howe et al. 2001). Of these germline *BMPR1A* mutations, 9 (64%) are located within exons 6–8 (exon 1 is the first coding exon; there are two other noncoding exons 5′ of exon 1), encoding part of the intracellular domain of the receptor (fig. 1), and, of these 9 mutations, 8 have occurred in the N-terminal 142 amino acids of the kinase domain, half of which are in close proximity to the ATP-binding site. There are no mutations located in or beyond the C-terminal half of the kinase domain. Interestingly, the two pro-

bands with mutations occurring in exons 5 and 6 both have macrocephaly and hypertelorism.

All but one of the nine mutations in the cytoplasmic domain are predicted to result in truncated receptors (fig. 1). The truncations all leave an intact transmembrane domain, such that the mutant receptors could be processed, to reach the plasma membrane, but are lacking all or part of the kinase domain. If the mutations in the cytoplasmic domain do result in truncated receptors, then these truncated receptors might be expected to bind ligand, but no signaling could occur. Thus, these intracellular-domain mutations might be predicted to act via dominant negative mechanisms. Family JP7/19 has a missense mutation in the middle of the kinase domain, C376Y. Residue 376 lies within the kinase domain, in close proximity to the active site, and is highly conserved among species—from *Caenorhabditis elegans* to mouse and rat. Four of the five mutations in the extracellular domain are predicted to result in truncated receptors. However, unlike the truncations in the cytoplasmic domain, two of the truncations would result in the lack of all or part of the signal peptide. The third truncation, S44X, results in a very short peptide without a transmembrane domain. Cysteine 124 lies in the cysteine-rich domain, which characterizes receptor kinases and is highly conserved across the TGFB family of type I and type II receptors, as well as across species (Kirsch et al. 2000). The ectodomain of BMPR1A has six intramolecular disulfide bridges between pairs of cysteines, which conformationally allows for BMP2 binding (Kirsch et al. 2000). Cysteine 124 is part of disulfide bond 4, and between the two cysteines forming this disulfide bridge lie nine key residues, which form part of the ligand-binding epitope. Loss of the sulfhydryl group at residue 124, as would be the case for this mutation, would therefore result in severe conformational alterations and in loss of the ability to bind ligand. The splice mutation IVS5-1g→t would be predicted to result in a receptor without a transmembrane domain. Thus, in general, extracellular-domain germline mutations—whether truncating or missense—together with the somatic second hit—as evidenced by LOH in the *BMPR1A* region in the majority of component tumors, both benign and malignant—might result in physical or functional lack of receptor. These observations contrast with those of Howe et al. (2001), who failed to detect LOH in component tumors from mutation-positive families. Our data demonstrating that *BMPR1A* behaves in accordance with the Knudson two-hit theory strongly suggest that *BMPR1A* encodes a tumor suppressor and likely also plays a gatekeeping function (Kinzler and Vogelstein 1998), much like SMAD4 itself (Woodford-Richens et al. 2000*b*).

Although the sample size is small, it would appear that, among the nine cytoplasmic-domain mutations, seven have occurred in familial cases of JPS whereas only two have occurred in isolated cases of JPS. In contrast, of the four extracellular-domain mutations, two occur in familial cases and three occur in isolated cases. Because of the small sample sizes of each subset, no statistical significance can be inferred. However, an interesting hypothesis to test in the future is that *BMPR1A* mutations that occur in the cytoplasmic domain and that are predicted to be dominant negative are associated with higher penetrance and with familial transmission. Because we have demonstrated LOH in component tumors from mutation-positive individuals—and if this hypothesis is correct—then the dominant-negative effect must act against other TGFB-receptor–family partners with which BMPR1A normally hetero-oligomerizes. Extracellular mutations that mainly result in haploinsufficiency, on the other hand, are associated equally with isolated and familial cases.

Because CS and BRRS lie within a single spectrum (Marsh et al. 1999), we chose to examine probands with CS/BRRS and CS/BRRS-like phenotype as one group. Only one such proband with CS/CS-like phenotype was found to harbor a *BMPR1A* mutation—specifically, A338D. This missense mutation occurs in the kinase domain—more specifically, immediately downstream of the kinase catalytic core—and in a residue that is highly conserved across species, from *C. elegans* to mouse and rat. Thus, if an acidic hydrophilic residue (aspartate) were substituted for a neutral nonpolar residue (alanine), the kinase catalytic core would be predicted to be disrupted. Although ligand binding might still be possible, this mutation could be predicted to result either in a loss of substrate specificity or in a receptor that might not be able to bind substrate.

Despite some initial confusion that germline *PTEN* mutations might be associated with rare cases of JPS (Olschwang et al. 1998), over the course of the past 4 years of clinical and molecular-epidemiologic analyses, it has become obvious that the presence of germline *PTEN* mutations defines CS and BRRS, regardless of the manner of clinical presentation (Eng and Ji 1998; Kurose et al. 1999; Marsh et al. 1999). This is germane for clinical cancer genetic practice, because the presence of *PTEN* mutations implies organ-specific surveillance of the patient and of his or her family. On the other hand, detection of a *MADH4* or a *BMPR1A* mutation should be considered diagnostic of JPS. In our opinion, families with CS/BRRS or CS/BRRS-like phenotype with *BMPR1A* mutations must therefore, on the basis of molecular data, be reclassified as having JPS.

Acknowledgments

This work was partially funded by American Cancer Society grant RPG98-211-01 (to C.E.), U.S. Army Breast Cancer Re-

search Program grant DAMD-17-00-1-0390 (to C.E.), and National Cancer Institute grant P30CA16058 (to The Ohio State University Comprehensive Cancer Center), as well as by grants from the Imperial Cancer Research Fund (to I.P.M.T.), from the European Union (to K.W.-R.), and from the Cancer Research Campaign (to R.S.H.). M.A. is a Fellow of the Wellcome Trust.

Electronic-Database Information

Accession numbers and the URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for JPS [MIM 174900], PJS [MIM 175200], CS [MIM 158350], and BRRS [MIM 153480])

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Human Molecular Genetics, 2001, Vol. 10, No. 18 1907–1913

Genetic model of multi-step breast carcinogenesis involving the epithelium and stroma: clues to tumour–microenvironment interactions

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Received April 18, 2001; Revised and Accepted June 21, 2001

ABSTRACT

Although numerous studies have reported that high frequencies of loss of heterozygosity (LOH) at various chromosomal arms have been identified in breast cancer, differential LOH in the neoplastic epithelial and surrounding stromal compartments has not been well examined. Using laser capture microdissection, which enables separation of neoplastic epithelium from surrounding stroma, we microdissected each compartment of 41 sporadic invasive adenocarcinomas of the breast. Frequent LOH was identified in both neoplastic epithelial and/or stromal compartments, ranging from 25 to 69% in the neoplastic epithelial cells, and from 17 to 61% in the surrounding stromal cells, respectively. The great majority of markers showed a higher frequency of LOH in the neoplastic epithelial compartment than in the stroma, suggesting that LOH in neoplastic epithelial cells might precede LOH in surrounding stromal cells. Furthermore, we sought to examine pair-wise associations of particular genetic alterations in either epithelial or stromal compartments. Seventeen pairs of markers showed statistically significant associations. We also propose a genetic model of multi-step carcinogenesis for the breast involving the epithelial and stromal compartments and note that genetic alterations occur in the epithelial compartments as the earlier steps followed by LOH in the stromal compartments. Our study strongly suggests that interactions between breast epithelial and stromal compartments might play a critical role in breast carcinogenesis and several genetic alterations in both epithelial and stromal compartments are required for breast tumour growth and progression.

INTRODUCTION

Breast cancer is the most common and second most lethal cancer in women in Western countries. Numerous studies have focused on the role of chromosome abnormalities and gene mutations in sporadic breast cancer, but to date no clear model of the critical events or delineation of primary abnormalities has emerged. Various chromosome arms have been observed to be affected by a high frequency of structural or numerical abnormalities (1–5). Although several of these chromosome arms appear to be the sites of putative tumour suppressor genes (TSGs), the number and identity of TSGs relevant for mammary carcinogenesis is unknown. At the molecular level, several somatic mutations in genes residing in these regions have been described (1,6–9). Despite this abundance of data, the relevance, role and timing of most of the described genetic abnormalities in sporadic breast cancer are still unclear. It is also not known whether specific mutations play relevant roles as causative factors or are the consequence of the general genomic instability and progression in breast tumours.

A few studies have previously demonstrated that loss of heterozygosity (LOH) identified at various chromosomal loci at high frequency in invasive cancer is already present in *in situ* carcinoma, atypical ductal hyperplasia, non-atypical hyperplasia of the breast, and perhaps adjacent normal epithelial cells, although these studies predated laser capture microdissection (LCM), hence, contamination from clearly malignant tissue cannot be excluded (10–15). These observations are also found in colonic adenomas (16), Barrett oesophageal metaplasia

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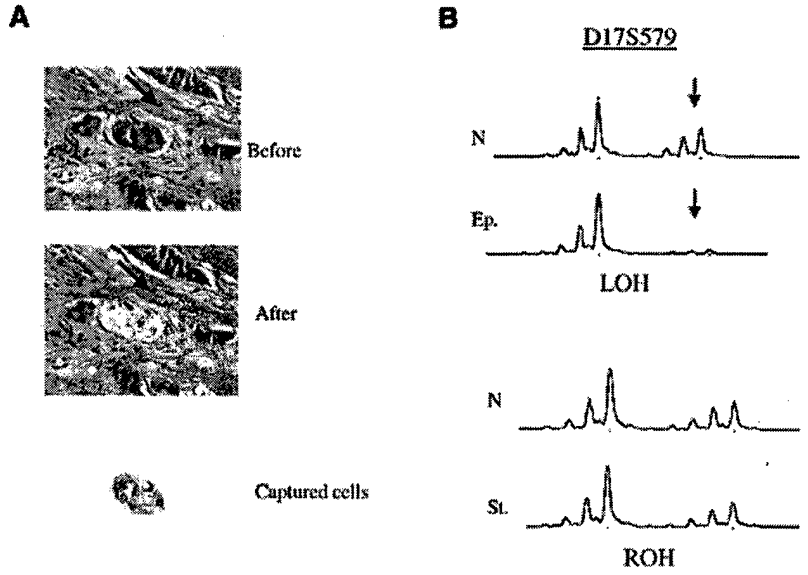

Figure 1. (A) LCM from breast cancer specimen. Captured cells are the neoplastic epithelial component. The surrounding stromal fibroblasts are immediately adjacent to the removed cells (arrow). (B) Illustrative examples of LOH (arrows) and retention of heterozygosity (ROH) at D17S579. N, normal cells; Ep., epithelial cells; St., stromal cells.

(17,18) and lung hyperplasias (19). These reports indicate that the majority of premalignant or precursor lesions share their LOH phenotypes with invasive disease in the same organs, providing novel biologic evidence that they are genetically and perhaps evolutionarily related. Nonetheless, until recently, any cancer such as breast cancer was treated as a single amorphous entity. Most such genetic studies were uniformly performed on the entire tumour without regard to its components, despite the fact that a few groups were quite aware of both epithelial and stromal components of tumours, and the cell biology of the tumour 'microenvironment' has been described for the last 20 years. Thus, until now, when a genetic alteration, be it intragenic mutation, regional amplification or deletion manifested by LOH, is attributed to a breast cancer, it is unclear if the alteration is actually occurring in the epithelial compartment, the surrounding stromal compartment or both.

The effects of the metabolism of the tumour stroma, locally as well as systemically, are largely unknown. Although the stroma has generally been considered a silent bystander during epithelial carcinogenesis, the concept that the microenvironment is central to maintenance of cellular function and tissue integrity provides the rationale for the idea that its disruption can contribute to neoplasia (20). Several studies performed *in vivo* and *in vitro* indicated that the growth and invasive potentials of carcinoma cells are influenced through interactions with host stromal cells (21–23). Despite these progressive cell biological studies, the precise genetic mechanisms leading to tumour progression remain unclear. Even less clear is the role of differential genetic alterations in the epithelial neoplastic component and its surrounding stroma.

Recently, Moinfar *et al.* (5) reported the high frequency of LOH in the mammary stroma with breast cancer. They examined 11 patients with ductal carcinoma *in situ*, including five cases with invasive ductal carcinoma, and found LOH in the stromal cells. Although these investigators identified frequent genetic alterations in the mammary stroma, each component of the breast carcinoma was manually microdissected; thus cross-contamination cannot be excluded. Further, they examined a limited number of samples and a limited number of microsatellite markers on only five chromosome arms. In this present study, we sought to systematically examine for genetic alterations in the epithelial and stromal components of invasive adenocarcinomas of the breast with the DNA extracted from cells from each compartment obtained by LCM. We found frequent LOH in both epithelial and/or stromal components of breast cancer and identified associations among LOH at various chromosomal regions, suggesting that genetic alterations in the epithelial and surrounding stromal cells are involved in the breast tumorigenesis through concurrent and independent pathways.

RESULTS

LCM of each specimen was performed to selectively obtain normal epithelial or stromal cells, carcinomatous epithelial cells and stromal cells surrounding the epithelial carcinoma (e.g. Fig. 1A). LOH at each of the 13 chromosomal regions was detected in epithelial and/or stromal compartments among the 41 invasive adenocarcinomas of the breast (e.g. Fig. 1B). Table 1 summarizes the frequencies of LOH observed at 13 loci in neoplastic epithelial and surrounding stromal cells. Among the 13 microsatellite markers examined, the LOH frequency ranged from 25% (9/36) at D3S1581 (3p14–q21) to 69% (22/32) at D17S796 (17p13) in the neoplastic epithelial compartment, and from 17% (6/36) also at D3S1581 to 61% (20/33) at D2S156 (2q34) in the surrounding stromal

Table 1. LOH frequencies and distribution in the epithelial and stromal cells

Chromosomal region	Marker	Cases with LOH in Ep[a]/informative cases	Cases with LOH in St[b]/informative cases	Cases with LOH in either Ep or St/ informative cases	Cases with LOH only in Ep	Cases with LOH only in St	Cases with LOH in both Ep and St	McNemar's test *P*-value (exact *P*-value)
1p36	D1S228	16/34 (47%)	10/34 (29%)	21/34 (62%)	11	5	5	0.1336 (0.2101)
2q34	D2S156	16/33 (48%)	20/33 (61%)	26/33 (79%)	6	10	10	0.3173 (0.4545)
3p14.2–p21.2	D3S1581	9/36 (25%)	6/36 (17%)	11/36 (31%)	5	2	4	0.2568 (0.4531)
3p24.3–p25.1	D3S1286	17/37 (46%)	14/37 (38%)	23/37 (62%)	9	6	8	0.4386 (0.6072)
6q25.3	D6S437	14/29 (48%)	15/29 (52%)	20/29 (69%)	5	6	9	0.7630 (1.000)
8p23.2	D8S264	21/32 (66%)	18/32 (56%)	25/32 (78%)	7	4	14	0.3657 (0.5488)
10q23.3	D10S1765	15/36 (42%)	10/32 (31%)	16/32 (50%)	6	5	5	0.7630 (1.000)
11q23	D11S912	19/37 (51%)	16/37 (43%)	25/37 (68%)	9	6	10	0.4386 (0.6072)
13q14	D13S155	18/33 (55%)	16/33 (48%)	23/33 (70%)	7	5	11	0.5637 (0.7744)
16q24.3	D16S413	20/31 (65%)	13/32 (41%)	23/31 (74%)	11	3	9	0.0325 (0.0574)
17p13	D17S796	22/32 (69%)	14/32 (44%)	24/32 (75%)	10	2	12	0.0209 (0.0386)
17q21	D17S579	17/32 (53%)	11/32 (34%)	21/32 (66%)	10	4	7	0.1088 (0.1796)
22q12.2–q13.1	D22S277	15/37 (41%)	15/37 (41%)	21/37 (57%)	6	6	9	1.000 (1.000)

[a]Ep, epithelium; [b]St, stroma.

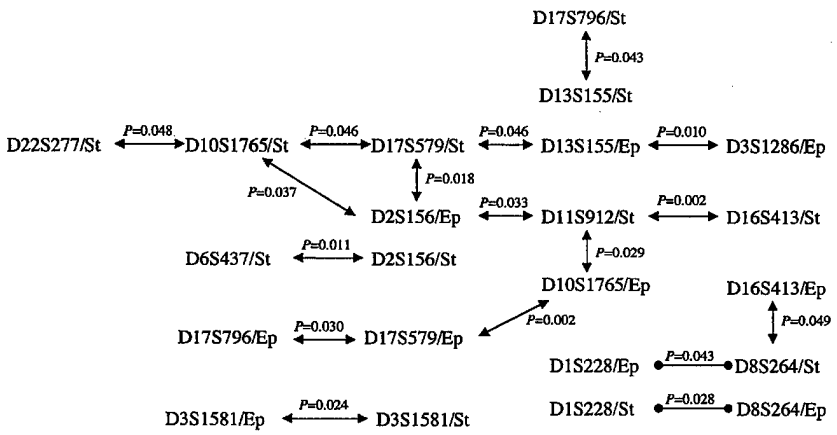

Figure 2. Positive and negative associations between markers in epithelial and stromal compartments of adenocarcinomas of the breast. Double-headed arrows denote positive correlations while double-knobbed lines denote negative correlations. Numbers above the arrows or lines are *P*-values (Fisher's exact test). Ep, epithelial cells; St, stromal cells.

component (Table 1). D2S156 (2q34) and perhaps D6S437 (6q25) were the only two markers demonstrating a higher frequency of LOH in the surrounding stromal compartment compared with the neoplastic epithelial cells. In contrast, the great majority of markers showed a higher frequency of LOH in the neoplastic epithelial compartment compared with the surrounding stromal cells (Table 1).

On further inspection of the differential LOH data, it can be noted that for certain markers, LOH predominates in the neoplastic epithelial compartment, for others, LOH predominates in the stromal compartment, and for yet other markers, it occurs in both compartments (Table 1). For instance, LOH at 16q24.3 (D16S413) was identified more frequently in only the neoplastic epithelial cells (11 tumours) than in only the stromal cells (three tumours; $P = 0.0325$, McNemar's test). The number of tumours that showed LOH at 17p13 (D17S796) only in stromal cells (two tumours) was significantly fewer than that having LOH at 17p13 in both epithelial and stromal cells or only in the neoplastic epithelial cells (12 or 10 tumours; $P = 0.0209$, McNemar's test).

We then looked for pair-wise associations of dependency or independency of particular genetic alterations with one another in either epithelial or stromal compartments (Fig. 2). We found that there were statistically significant associations in 17 pairs

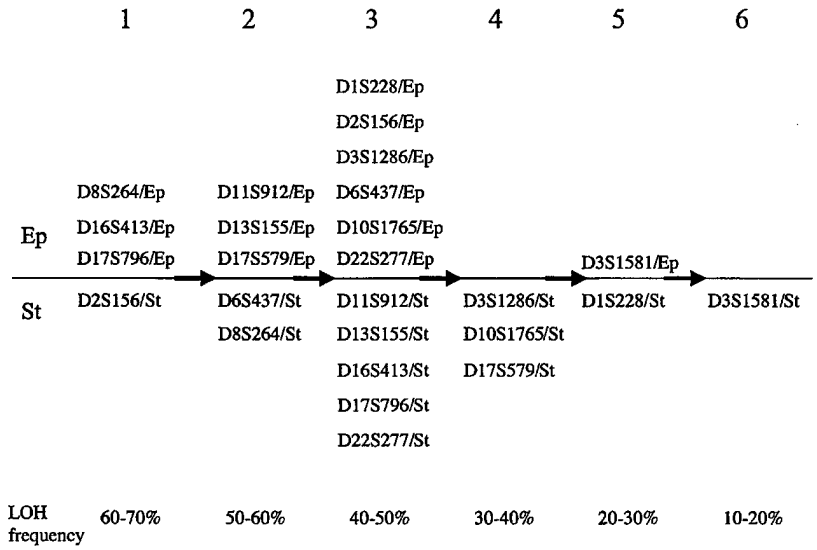

Figure 3. Proposed genetic model of multistage, stepwise carcinogenesis in the breast involving the epithelial and stromal compartments. Ep, epithelial cells; St, stromal cells.

(17/325; 5.3%) of markers (Fig. 2). Fifteen pairs (15/17; 88%) showed positive associations and two pairs (2/17; 12%) showed negative associations (Fig. 2). LOH at four markers, D10S1765, D11S912 and D17S579 in the surrounding stromal compartment and D2S156 in the neoplastic epithelial compartment, were associated with three or more sites of LOH (Fig. 2). Of note, LOH at D10S1765 in the stromal compartment was associated with LOH at D17S579 and D22S277 in the stromal compartments and also with LOH at D2S156 in the neoplastic epithelial compartments. LOH at D17S579 in the stromal compartment, in turn, was found to be associated with LOH at D2S156 and D13S155 in the neoplastic epithelial compartment and at D10S1765 in the surrounding stromal compartment. In contrast, LOH at D8S264 (8p23.2) in the neoplastic epithelial cells is negatively correlated with LOH at D1S228 (1p36) in the surrounding stromal cells. Interestingly, LOH at D8S264 in the surrounding stromal compartment is negatively correlated with LOH at D1S228 in the neoplastic epithelial compartment.

DISCUSSION

In this present study, we have found frequent LOH in both neoplastic epithelial and surrounding stromal cells of invasive adenocarcinomas of the breast. In our series of 41 breast cancer samples, we identified LOH in the neoplastic epithelial compartment, ranging from 25% (9/36) to 69% (22/32), and in the stromal compartment, ranging from 17% (6/36) to 61% (20/33), respectively. Of note, the great majority of markers demonstrated a higher frequency of LOH in the neoplastic epithelial compartment compared with the surrounding stromal cells (Table 1). Further inspection of the differential LOH data indicated that the frequency of LOH only in the stromal compartment occurred among fewer tumours than that in both neoplastic epithelial and surrounding stromal compartments, or that in only the neoplastic epithelial cells (Table 1).

In the field of human cancer genetics, it has been shown that markers with the highest frequency of LOH represent those with the earliest genetic alterations, the so-called first 'hits', and the one with the lowest frequency of LOH represents the latest 'hit' in carcinogenesis (16). Thus, under this assumption, from our data, we can propose a genetic model of multistage, stepwise carcinogenesis in the breast, according to the relative frequencies of LOH in the epithelial and stromal compartments (Fig. 3). Our proposed model encompasses data that shows a higher frequency of LOH in breast epithelial cells occurs earlier than in the stromal compartment (Fig. 3). Although Moinfar *et al.* (5) worked without the advantage of LCM and used markers representing only five chromosomal regions and a sample size of 11, their observations of relative frequencies of LOH in the neoplastic epithelium (occurring in 1/4 to 3/3 tumours) compared with presumably surrounding stroma (occurring in 1/4 to 4/5 tumours) might be interpreted as concurrent with ours. These data together with our observations suggest that genetic alterations in the epithelial compartment, at least in some chromosomal regions, precede the genetic changes in the surrounding stromal cells. If in fact we may extrapolate that each region of LOH represents at least one putative TSG, then it is possible that the same putative gene involved in epithelial carcinogenesis plays some role in the stroma at a later stage, with the possible exception of D2S156. In our study, the earliest genetic alterations occurred at D8S264, D16S413 and D17S796 in the epithelium as well as D2S156 in the stroma (Fig. 3). It is almost certain that the D17S796 marker represents *TP53*, and indeed, *TP53* alterations have been noted amongst the most frequent and earliest somatic alterations in prior studies involving 'whole' breast carcinomas (24,25). The regions of D8S264 and D16S413 have yet to yield convincing TSGs involved in breast carcinogenesis. Our data would strongly support the existence of one or more TSGs residing in these two regions which when

mutated participate in the initiation of cancer within the breast epithelium. Of interest, LOH at D2S156 in the stromal cells is also scored as an early event (Fig. 3). This has not been a region noted to have LOH in whole breast cancers. Nonetheless, our data suggest that there will be at least one important gene residing in that interval which plays a prominent role in the initiation of breast carcinogenesis, possibly from a microenvironmental or 'landscaper' point of view (26). The genetic model proposed (Fig. 3) assumes for simplicity that frequency of occurrence reflects temporal sequence. This in turn assumes an equivalent effect on tumorigenesis and early progression between all alterations. However, it is recognized that an alternative possibility is that some alterations may be dominant while others may require the cooperation of parallel or multiple complex alterations at other sites to facilitate progression, and that this would influence the prevalence of the alteration in advanced tumours. In the case of these different assumptions, a lower frequency of stromal alterations could reflect the fact that stromal alterations are not dominant and only exert an indirect effect on the adjacent epithelium, or only exert an effect in collaboration with others, to influence the overall process of tumorigenesis.

The apparent asynchronous LOH at each marker between epithelium and stroma might suggest that while the neoplastic epithelium is clonal, as is the stroma, these observations may support one viewpoint that epithelium and stroma derive from different cellular origins. However, there are advocates of a common cellular origin of both epithelium and stroma (27). If this latter is true, then in the context of our observations, the LOH in epithelium and stroma occurred after the divergence of epithelial and stromal cell from the presumed common cell.

Despite a relatively small sample size, we were able to examine pair-wise associations between regions and compartments where LOH occurred (Fig. 2). For example, LOH at D17S579 in the neoplastic epithelial cells was associated with LOH at D17S796 and D10S1765 in the neoplastic epithelial compartments. These chromosomal loci contain several putative TSGs. The polymorphic markers, D17S579 (17q21), D17S796 (17p13) and D10S1765 (10q23.3), are in proximity to the *BRCA1*, *TP53* and *PTEN* genes, respectively. Previous reports have identified that there are significant associations between LOH of *BRCA1* and *TP53* (28,29) or *PTEN* gene (28) in sporadic heterogeneous breast cancer samples. Crook *et al.* (30) found a high proportion of 'whole' breast and ovarian tumours from *BRCA1* mutation carriers had *TP53* mutations. Our proposed genetic model does suggest that LOH at D17S796 in the neoplastic epithelial cells is the earliest hit, LOH at D17S579 in the neoplastic epithelial cells is the second one, then LOH at D10S1765 in the neoplastic epithelial cells is the third hit in these consequences (Table 1 and Fig. 3). The association between LOH of *BRCA1* and *PTEN* is, therefore, one of the genetic alterations that might be expected to occur as a consequence of the loss of *BRCA1* and *TP53*. LOH at D2S156 in the neoplastic epithelial compartment was associated with LOH at three markers, D10S1765, D11S912 and D17S579, in the surrounding stromal compartments. The reciprocal interaction between epithelial and stromal cells plays a key role in the morphogenesis, proliferation and differentiation of epithelial cells (31–33). Most of the intercellular substances, extracellular matrix (ECM) molecules that are required for tumour growth and progression are produced by the stromal cells (34). It is well demonstrated that altered gene expression occurs between normal and neoplastic breast stroma (35) and that stromal cells play a critical role in the production and possible dissolution of the ECM (36,37). Thus, genetic alterations in the stromal cells may change the interaction between epithelial cells and ECM molecules and influence the tumour invasion and dissemination (22,23). Our results suggest that LOH at 2q34 (D2S156) might precede LOH of three chromosomal loci in the stromal compartments (Figs 2 and 3). Therefore, we hypothesized that loss of a putative TSG on 2q34 might play an important role in genetic alterations of stromal compartments, which in turn might influence tumour invasion and dissemination through ECM remodelling. If this hypothesis is correct, then we can further hypothesize that genetic alterations in the stroma might predict for poorer prognosis due to increased tumour invasiveness.

In summary, we have found frequent LOH in both neoplastic epithelial and surrounding stromal compartments in invasive adenocarcinomas of the breast and statistically significant associations among the LOH at various chromosomal regions. We also propose a multi-step genetic model of breast carcinogenesis involving epithelium and stroma, which can help build further hypotheses and guide future studies of reciprocal interactions between breast neoplastic epithelial and stromal cells in tumour initiation, progression, invasion and metastases. Such studies might eventually lead to novel therapeutic strategies, which selectively target epithelium or stroma.

MATERIALS AND METHODS

Breast cancer samples

Forty-one archival (formalin-fixed and paraffin-embedded) tissues that were distinct cases of clinically sporadic primary invasive adenocarcinomas of the breast were used. Twenty-six samples were obtained from the National Cancer Institute of Canada Manitoba Breast Tumour Bank and 15 were from the Department of Pathology of The Ohio State University.

Microdissection of tissue and DNA extraction

Microdissection of carcinomatous epithelial cells, surrounding stromal cells, and normal epithelial or stromal cells from fixed, paraffin-embedded sections of breast was performed using an Arcturus PixCell II Laser Capture microdissecting microscope (Arcturus Engineering Inc., Mountain View, CA). This system utilizes a transparent thermoplastic film applied to the surface of the tissue section on standard histopathology slides. The breast cancer epithelial, surrounding stromal, and normal epithelial or stromal cells to be microdissected were identified and targeted through a microscope, and a narrow (~15 uM) carbon dioxide laser-beam pulse specificity activated the film above these cells. The resulting strong focal adhesion allowed selective procurement of only the targeted cells (38) (Fig. 1A). The cells removed in Figure 1A are the neoplastic epithelial component. The surrounding stromal fibroblasts are immediately adjacent to the removed cells (Fig. 1A, arrow). It is acknowledged that while LCM minimizes cross-contamination of cell types, it does not guarantee against it. However, the very 'clean' LOH (virtually all or none) which we have obtained does suggest that any cross-contamination is not significant.

DNA from microdissected tissue was extracted in 50 µl of solution containing 0.04% proteinase K, 1% Tween-20, 10 mM Tris–HCl (pH 8.0), and 1 mM EDTA at 37°C overnight followed by heat inactivation at 95°C for 10 min.

LOH analysis

For purposes of this study, genomic DNA, extracted from paraffin embedded tissues, served as template for PCR amplification of 13 microsatellite markers selected from a comprehensive genetic map of the human genome (39). Fluorescent-labelled polymorphic markers, including D1S228 (1p36), D2S156 (2q34), D3S1581 (3p14.2–p21.2), D3S1286 (3p24.3–p25.1), D6S437 (6q25.3), D8S264 (8p23.2), D10S1765 (10q23.3), D11S912 (11q23), D13S155 (13q14), D16S413 (16q24.3), D17S796 (17p13), D17S579 (17q21) and D22S277 (22q12.2–q13.1), were used for this analysis. All subsequent PCRs were carried out using 0.5 µM each of forward and reverse primer in 1× PCR buffer (Qiagen, Valencia, CA), 1.5 mM $MgCl_2$ (Qiagen), 1× Q-buffer (Qiagen), 1.25 U of HotStar Taq polymerase (Qiagen) and 200 µM of each dNTP (Gibco, Gaithersburg, MD) in a final volume of 25 µl. After a denaturation at 95°C for 14 min, reactions were subjected to 40 cycles of 94°C for 1 min, 55–60°C for 1 min, and 72°C for 1 min followed by 10 min at 72°C. PCR reactions and genotyping were repeated at least a second time to confirm the data. Amplified PCR products were separated by electrophoresis through 6% denaturing polyacrylamide gels, and the signal was detected with an Applied Biosystems model 377×l semi-automated DNA sequencer (Applied Biosystems, Perkin-Elmer Corp., Norwalk, CT). The results were analysed by automated fluorescence detection using the GeneScan collection and analysis software (GeneScan, Applied Biosystems). Scoring of LOH was initially performed by inspection of the GeneScan analysis output. A conservative ratio of peak heights of alleles between germline DNA and somatic DNA ≥1.9:1 were used to define LOH in this study (40).

Statistical analysis

Comparisons for statistical significance were performed by using either the standard Fisher's exact test (2-tailed) or the McNemar's test for matched pairs at the $P = 0.05$ level of significance. McNemar's test was used when interest focused on differences in proportions of patients with LOH in either stromal or epithelial cells, but not both. This test determines whether, in these cases of discordance, there are a disproportionate number of patients with LOH in one of the two sites. McNemar's test is used in recognition of the fact that the stromal and epithelial cells are taken from the same breast tissue and, hence, are matched. However, the Fisher's exact test was also employed because it is unclear from a biological point of view whether each data point (i.e. LOH at any one marker) is dependent on the next (i.e. LOH at other markers).

ACKNOWLEDGMENTS

We thank Fred Wright and Sandya Liyanarchchi for providing statistical assistance. Part of this work was performed in the Laser Capture Microdissection core facility in the Tissue Procurement Shared Resources Service of the Comprehensive Cancer Centre. This work was supported in part by the Jimmy V Golf Classic Award for Basic and Clinical Cancer Research from the V Foundation (to C.E.) and a grant from the National Cancer Institute, Bethesda, MD (P30CA16058 to The Ohio State University Comprehensive Cancer Centre).

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Frequent somatic mutations in *PTEN* and *TP53* are mutually exclusive in the stroma of breast carcinomas

Published online 15 October 2002; doi:10.1038/ng1013

We have recently shown that loss of heterozygosity of specific markers, including those at 10q23, 17p13–p15 and 16q24, can occur in the stromal and epithelial compartments of primary invasive breast carcinomas. Here, we demonstrate high frequencies of somatic mutations in *TP53* (encoding tumor protein p53) and *PTEN* (encoding phosphate and tensin homolog) in breast neoplastic epithelium and stroma. Mutations in *TP53* and *PTEN* are mutually exclusive in either compartment. In contrast, mutations in *WFDC1* (16q24, encoding WAP four-disulfide core domain 1) occur with low frequency in the stroma.

In solid tumors, including breast carcinomas, genetic alterations manifested by loss of heterozygosity (LOH) of markers suggest that putative tumor-suppressor genes may influence carcinogenesis. Despite extensive study of multiple regions of LOH, few such regions have yielded convincing novel genes[1]. So far, the great majority of LOH studies have been performed on 'whole' breast cancers and rarely on the epithelium alone. We recently found that LOH of selected markers can occur in breast tumor stroma[2]. In the context of the Knudson two-hit theory and our observations of LOH in stroma, we sought to determine if there exist mutations in tumor-suppressor genes within regions of LOH in stroma. We selected breast cancer as our model, and examined three genes within three regions that we have already shown to have LOH in breast cancer stroma[2] (S.M. and C.E., unpublished observations). We selected the tumor-suppressor gene *TP53* because it has already been shown to have a somatic mutation frequency of roughly 20–25% (see p53 Database, URL below), matching the high frequency of LOH on 17p13, in whole breast cancers and the epithelial neoplastic component. We selected *PTEN* on 10q23 because whereas the frequency of LOH ranges from 30–50% in primary whole breast carcinomas, the frequency of mutation is substantially less than 5% (reviewed in ref. 3). We selected a third gene, *WFDC1* (encoding ps20 expressed in rat prostate cancer stroma[4]), within 16q24.3, a common region of LOH previously described in whole breast carcinomas that has not previously been found to contain a breast tumor–suppressor gene.

We isolated genomic DNA obtained by laser-capture microdissection from breast cancer epithelium and surrounding stroma as described previously[2]. We analyzed DNA from each compartment and the germ line for mutations in *TP53* and *WFDC1* by direct sequence analysis (primers available on request) and for mutations in *PTEN* using denaturing gradient gel electrophoresis and sequence analysis as previously described[5]. We identified somatic *TP53* mutations in 25 of 50 (50%) breast cancer samples (obtained under Institutional Review Board approval 01H0384; Table 1*a*). Of these 25 samples, 11 had *TP53* mutations in the epithelium alone, 9 in the stroma alone and 5 in both (Table 1*a*). In all but three instances, mutations in *TP53* (whether in the epithelium or stroma) were accompanied by LOH at *D17S796* (Table 1*a*). One sample (tumor 104, Table 1*a*) did not have LOH at 17p13 but had two mutations in *TP53*. Consistent with previous somatic mutational spectra for *TP53* in various solid tumors, four mutations were truncating and the rest were missense, with hot spots at codons 248 and 273 (see URL below). Of 37 total somatic mutations in epithelium and stroma, 25 have been observed previously either in the germ line of individuals with Li–Fraumeni syndrome or as somatic mutations in neoplasias, and 12 have not been observed previously. Of these 12, 9 were found in codons that were previously found to be mutated (Table 1; see URL below). In two of the nine samples in which we found somatic mutations in *TP53* in both the epithelium and stroma, the mutations were discordant between the two compartments (Table 1*a*, samples 17 and 55; Fig. 1*a*). Our observations contrast with a previous report[6] that did not find mutations in *TP53* in stroma or epithelium in 15 breast carcinomas, probably owing to the sample size, incomplete mutation screening (exons 5–8), less sensitive techniques (single-strand conformational polymorphisms), population differences (German versus American) or some combination of these factors.

We found that 15 of 50 (30%) breast carcinomas had somatic mutations in *PTEN* in either neoplastic epithelium or stroma (Table 1*b* and Fig. 1*b*,*c*). Unlike the compartmental pattern observed for mutations in *TP53*, mutations in *PTEN* were not

Table 1 • Somatic mutations in *TP53* and *PTEN* in the epithelium and stroma of invasive breast carcinomas

a Mutations in *TP53*

	Tumor	LOH at *D17S796*	Exon	Mutation	Codon altered
1	ep	LOH	5	406C→T	Gln136X
			5	535C→T	His179Tyr
	st	ROH	wt	–	–
2	ep	ni	wt	–	–
	st	ni	7	737G→A	Arg248Gln
3	ep	LOH	8	845G→C	Arg282Pro
	st	ROH	wt	–	–
6	ep	LOH	wt	–	–
	st	ROH	8	856G→T	Glu286X
7	ep	ni	4	166G→A	Glu56Lys
	st	ni	wt	–	–
9	ep	ni	7	743G→A	Arg248Gln
	st	ni	wt	–	–
13	ep	ni	wt	–	–
	st	ni	5	377A→T	Tyr126Phe
			8	856G→T	Gln286X
15	ep	LOH	wt	–	–
	st	LOH	10	1100G→A	Ser367Asn
17	ep	ni	6	659A→G	Tyr220Cys
	st	ni	7	734G→A	Gly245Asp
19	ep	LOH	5	393–395delCAA	in-frame deletion
	st	ROH	wt	–	–
22	ep	ROH	10	1082G→T	Gly361Val
	st	ROH	wt	–	–

Table 1 • (continued)

	Tumor	LOH at *D17S796*	Exon	Mutation	Codon altered
23	ep	LOH	5	535C→A	His179Asn
	st	ROH	wt	–	–
26	ep	LOH	7	742C→T	Arg248Trp
	st	ROH	7	742C→T	Arg248Trp
52	ep	LOH	8	818G→C	Arg273Pro
	st	LOH	wt	–	–
54	ep	ROH	2	37C→T	Pro13Ser
	st	ROH	wt	–	–
55	ep	LOH	2	4G→C	Glu2Gln
	st	ni	6	667C→G	Pro222Ala
56	ep	LOH	7	742C→T	Arg248Trp
	st	LOH	wt	–	–
58	ep	LOH	9	950A→G	Gln317Arg
	st	LOH	ni	–	–
60	ep	LOH	wt	–	–
	st	ROH	4	305C→T	Thr102Ile
			5	427G→T	Val143M
				529C→T	Pro177Ser
				548C→T	Ser183Leu
65	ep	LOH	wt	–	–
	st	LOH	8	788A>G	Asn263Ser
104	ep	ROH	4	187G→T	Ala63Ser
			4	293C→T	Pro98Leu
	st	LOH	wt	–	–
105	ep	LOH	8	817C→T	Arg273Cys
	st	nd	8	817C→T	Arg273Cys
119	ep	nd	wt	–	–
	st	nd	6	659A→G	Tyr220Cys
121	ep	nd	wt	–	–
	st	nd	6	637C→T	Arg213Ser
126	ep	ni	9	946C→T	Pro316Ser
	st	ni	wt	–	–

b Mutations in *PTEN*

	Tumor	LOH at *D10S1765*	Exon	Mutation	Codon altered
8	ep	ROH	wt	–	–
	st	ROH	7	800delA	frameshift
10	ep	ROH	wt	–	–
	st	ROH	8	963–8delA	frameshift
11	ep	ROH	wt	–	–
	st	ROH	9	1021T→G	Phe341Val
12	ep	ROH	wt	–	–
	st	ROH	9	1021T→G	Phe341Val
13	ep	ROH	9	1061C→G	Pro354Arg
	st	ROH	wt	–	–
14	ep	ROH	wt	–	–
	st	ROH	5	–	Gln110X
20	ep	LOH	5	373–375delA	frameshift
	st	ROH	wt	–	–
54	ep	ROH	wt	–	–
	st	ROH	2	IVS2–9T→C	aberrant splice[a]
56	ep	ROH	3	181G→C	Asp61His
	st	ROH	wt	–	–
62	ep	LOH	1	IVS1+62A/G	cryptic splice[a]
	st	LOH	wt	–	–
63	ep	ROH	5	IVS4–30delA	cryptic splice[a]
	st	ROH	wt	–	–
64	ep	ni	7	758T→A	Ile253NAsn
	st	ni	wt	–	–
114	ep	nd	5	455T→C	Leu152Pro
	st	nd	5	389G→C	Arg130Prp
126	ep	nd	5	316G→T	Glu106X
	st	nd	wt	–	–

[a]Data not shown. The LOH data presented here have been previously used for *en bloc* analysis[2] except for new LOH data represented by samples 104, 105, 114, 119, 121 and 126. All exons, exon–intron boundaries and at least 20 bp of flanking intronic sequences for *TP53*, *PTEN* and *WFDC1* were subjected to mutation analysis. ep, epithelium; LOH, loss of heterozygosity; nd, not done; ni, not informative; ROH, retention of heterozygosity; st, stroma; wt, wildtype.

found in both compartments in any single sample, except for sample 114. Mutations in *TP53* and *PTEN* were mutually exclusive. In further contrast with *TP53*, mutations in *PTEN* were not accompanied by LOH (Table 1, Fig. 1*b*). We found mutations in *WFDC1* in only 3 of 50 (6%) samples, 2 in the stroma alone (resulting in a Gly9Asp amino-acid substitution and LOH, and Pro211Ser and Lys217Arg substitutions, respectively) and 1 in the epithelium alone (resulting in a Pro167Ser substitution, without LOH). All three samples also harbored somatic mutations in *TP53* in the same compartments (tumors 2, 22 and 60, Table 1). As a negative control, we examined the gene *SDHB*, an eight-exon mitochondrial complex II gene that can act as a tumor-suppressor gene in paraganglioma genesis but is not known to be a breast tumor–suppressor gene, by sequence analysis, and found no somatic variants (data not shown).

So far, genetic alterations in carcinomas, including breast cancers, have been attributed to the neoplastic epithelial cells. In this study, we found somatic mutations in *TP53*, *PTEN* and *WFDC1* with varying frequencies and distribution in the neoplastic epithelium alone, the surrounding stroma alone or both compartments of invasive breast carcinomas. Notably, when mutations were found in both compartments, at least three tumors showed discordant mutations (two in *TP53*, one in *PTEN*) between epithelium and the respective stroma. When identical somatic mutations in *TP53* occur in both the epithelium and stroma, accompanied by LOH of the same allele (as was the case for two samples), there can be at least two interpretations. (i) The mutation and LOH occurred in a single precursor cell type, which differentiated and clonally expanded into both epithelium and stroma. (ii) Although we used the laser-capture microdissection technique, there could have been cross-contamination between the two compartments. The latter is unlikely (especially in view of the isolated mutations in either compartment in many tumors across the three genes), and the observation of discordant mutations in epithelial and stromal compartments in three tumors argues against cross-contamination. This may suggest that, at least in certain subsets of breast cancers, somatic genetic alterations can occur as independent events in epithelium and stroma.

These hypotheses are supported by the compartmental pattern observed for somatic mutations in *PTEN* in this same set of tumors. Overall, mutations in *PTEN* occurred either in the neoplastic epithelium alone or in the stroma alone, arguing

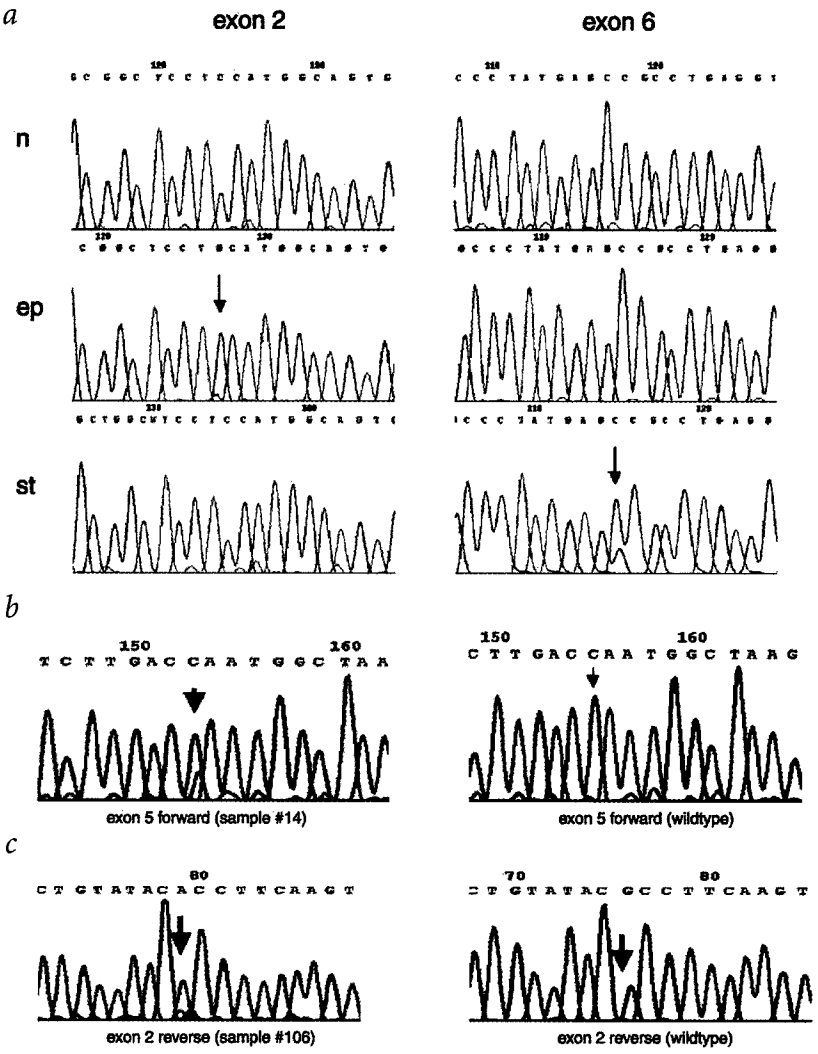

Fig. 1 Somatic mutations in *TP53* or *PTEN* in epithelium or stroma of breast carcinoma samples. *a*, Discordant somatic mutations in *TP53* in the epithelium and stroma. Sample 55 showed LOH at *D17S796* in the epithelium (ep); a hemizygous somatic Glu2Gln (exon 2, reverse sequence) mutation in the neoplastic epithelium (n); and a somatic Pro222Ala (exon 6, reverse sequence) mutation in the tumor stroma (st). *b*, Sample 14 showed a somatic heterozygous mutation in *PTEN* (Gln110X) in the stroma, not accompanied by LOH. *c*, Sample 106 showed a hemizygous conservative sequence variant (132C→T) in *PTEN* with accompanying loss of the opposite allele, suggesting clonality.

against cross-contamination and suggesting that genetic alterations might indeed occur independently in either compartment. The fact that mutations in *PTEN* occurred in either compartment in one-third of the tumors examined might explain the discrepancy between observed frequencies of mutation (substantially less than 5%) and LOH (30–40%) in whole breast carcinomas. The overall observation that mutations in *PTEN* and *TP53* do not co-occur in the same sample or in the same compartment corroborates our statistical model showing that LOH in the vicinity of *PTEN* (*D10S1765*) in either compartment is not directly associated with LOH in the *TP53* region (*D17S796*; ref. 2). Taken together with the functional data, which suggest that p53 partially regulates *PTEN* transcription[7], our observations might suggest that in breast cancers, the interplay between p53 and PTEN in this manner is germane to pathogenesis.

Our observations provide genetic evidence that mutations in stroma could contribute to the epithelial–stromal cross-talk in breast carcinogenesis. We also show that different mechanisms of gene silencing can occur in epithelium and stroma. For *TP53*, mutation and LOH is the major mechanism; for *PTEN*, the first genetic hit (mutation or LOH) may be accompanied by non-genetic silencing[8], but if it is not, it may act in a haploinsufficient mechanism where loss of one allele is sufficient for tumorigenesis. We would also suggest that such mechanisms are not limited to breast cancer, and that somatic mutations in the stroma of solid tumors might be universal.

URL. Institut Curie's website at http://p53.curie.fr/ contains a database of germline mutations in *TP53* in Li–Fraumeni syndrome as well as a database of somatic mutations in *TP53* in a variety of sporadic neoplasias, including those of the breast. The somatic database holds such information as frequency of mutations overall for each neoplasia type and frequencies and mutational spectra at each codon or nucleotide.

Acknowledgments

We are grateful to M. Ostrowski and G. Leone for helpful discussions and to C. Morrison, M. Prasad and A. Adewale for assistance. This work was supported in part by the Jimmy V Golf Classic Award for Translational Cancer Research from the V Foundation (to C.E.), the American Cancer Society (to C.E.), the Department of Defense US Army Breast Cancer Research Program (to P.H.W. and C.E.) and the National Cancer Institute (to The Ohio State University Comprehensive Cancer Center).

Competing interests statement

The authors declare that they have no competing financial interests.

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Received 20 June; accepted 5 September 2002.

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Am. J. Hum. Genet. 73:404–411, 2003

Report

Germline *PTEN* Promoter Mutations and Deletions in Cowden/Bannayan-Riley-Ruvalcaba Syndrome Result in Aberrant PTEN Protein and Dysregulation of the Phosphoinositol-3-Kinase/Akt Pathway

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Germline intragenic mutations in *PTEN* are associated with 80% of patients with Cowden syndrome (CS) and 60% of patients with Bannayan-Riley-Ruvalcaba syndrome (BRRS). The underlying genetic causes remain to be determined in a considerable proportion of classic CS and BRRS without a polymerase chain reaction (PCR)-detectable *PTEN* mutation. We hypothesized that gross gene deletions and mutations in the *PTEN* promoter might alternatively account for a subset of apparently mutation-negative patients with CS and BRRS. Using real time and multiplex PCR techniques, we identified three germline hemizygous *PTEN* deletions in 122 apparently mutation-negative patients with classic CS ($N=95$) or BRRS ($N=27$). Fine mapping suggested that one deletion encompassed the whole gene and the other two included exon 1 and encompassed exons 1–5 of *PTEN*, respectively. Two patients with the deletion were diagnosed with BRRS, and one patient with the deletion was diagnosed with BRRS/CS overlap (features of both). Thus 3 (11%) of 27 patients with BRRS or BRRS/CS-overlap had *PTEN* deletions. Analysis of the *PTEN* promoter revealed nine cases (7.4%) harboring heterozygous germline mutations. All nine had classic CS, representing almost 10% of all subjects with CS. Eight had breast cancers and/or benign breast tumors but, otherwise, oligo-organ involvement. PTEN protein analysis, from one deletion-positive and five *PTEN*-promoter-mutation–positive samples, revealed a 50% reduction in protein and multiple bands of immunoreactive protein, respectively. In contrast, control samples showed only the expected band. Further, an elevated level of phosphorylated Akt was detected in the five promoter-mutation–positive samples, compared with controls, indicating an absence of or marked reduction in functional PTEN. These data suggest that patients with BRRS and CS without PCR-detected intragenic *PTEN* mutations be offered clinical deletion analysis and promoter-mutation analysis, respectively.

Cowden syndrome (CS [MIM 158350]) is an autosomal dominant disorder characterized by multiple hamartomas affecting derivatives of all three germ layers and by an increased risk of breast, thyroid, and endometrial neo-

Received March 27, 2003; accepted for publication May 22, 2003; electronically published July 3, 2003.

0002-9297/2003/7302-0018$15.00

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plasia (Eng 2000). *PTEN/MMAC1/TEP1* (MIM 601728) is a tumor-suppressor gene located at 10q23.3, which antagonizes the phosphoinositol-3-kinase (PI3K)/Akt pathway (reviewed by Waite and Eng [2002]). Proper PTEN signaling leads to G1 cell–cycle arrest and/or apoptosis (reviewed by Waite and Eng [2002]). When ascertained strictly by International Cowden Consortium Operational Diagnostic Criteria, ~80% of patients with CS demonstrate germline *PTEN* mutations (Liaw et al. 1997; Marsh et al. 1998). In addition, ~60% of individuals with Bannayan-Riley-Ruvalcaba syndrome (BRRS [MIM 153480])—another autosomal dominant hamartoma syndrome characterized by a classic triad of macrocephaly, lipomatosis, and speckled penis—carry germline *PTEN* mutations (Marsh et al. 1997, 1998, 1999). Subsequently, the clinical spectrum of disorders associated with germline *PTEN* mutations has expanded to include seemingly disparate syndromes, such as Proteus syndrome (MIM 176920), Proteus-like syndromes, and VATER association with macrocephaly (Zhou et al. 2000*b*, 2001; Reardon et al. 2001; Smith et al. 2002).

The underlying genetic causes remain undetermined in 20% and 40%, respectively, of individuals with classic CS and BRRS in whom no mutations have been detected by conventional mutation-detection techniques (reviewed by Waite and Eng [2002]). Because CS is believed to be without genetic heterogeneity (Nelen et al. 1996), we hypothesized that apparently *PTEN*-mutation–negative CS and BRRS may be attributed to large gene rearrangements and deletions, which cannot be detected by conventional techniques, and promoter mutations. To test our hypotheses, therefore, we used a combination of real-time quantitative multiplex PCR analysis, fluorescent-based semiquantitative PCR assay, and microsatellite analysis to define and characterize *PTEN* and regional deletions in a large series of probands with CS and BRRS previously found not to have intragenic *PTEN* mutations. Further, deletion-negative samples were subjected to sequence analysis of the promoter region of *PTEN*. Finally, we biochemically characterized the potential pathogenicity of the deletion and promoter mutations.

After written informed consent was received, DNA from peripheral blood was obtained from 122 unrelated individuals diagnosed with CS ($N = 95$; 79%), according to the International Cowden Consortium diagnostic criteria (Eng 2000), or BRRS ($N = 27$; 21%), by clinical definition (Gorlin et al. 1992). Real-time quantitative PCR analysis was performed by use of the ABI 7700 Sequence Detector System (ABI/Perkin Elmer), as described elsewhere (Sieber et al. 2002). *PTEN* exons 1 and 5 were chosen as targets for the real-time quantitative PCR assay, whereas the remaining exons were analyzed by use of fluorescent-based semiquantitative multiplex PCR assay. Human *RET* exon 8 was chosen as the internal control. Primer and probe sequences are listed in table 1. The raw data obtained from real-time PCR was analyzed by use of the comparative C_T method (as described in User Bulletin No. 2, ABI/Perkin Elmer), with normalization to the internal control, *RET*. Samples without

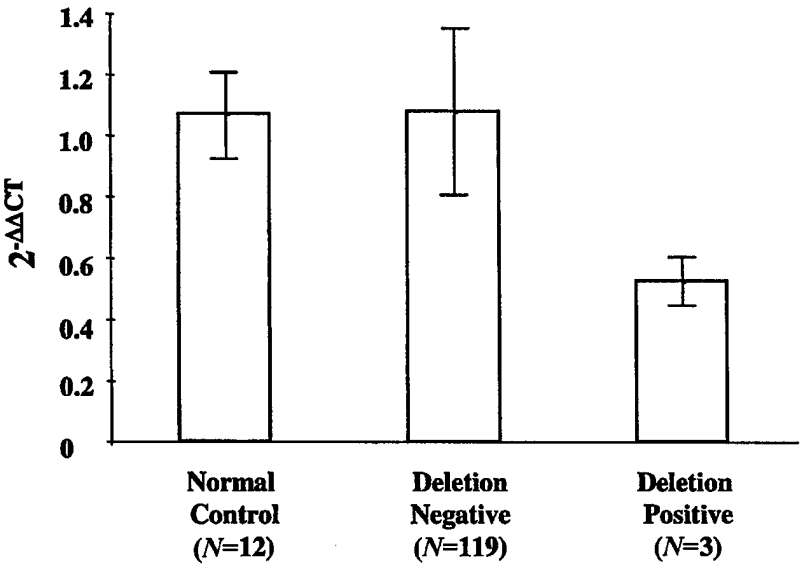

Figure 1 Real-time quantitative multiplex PCR results for 12 normal control subjects and 122 apparently mutation-negative individuals with CS and/or BRRS at *PTEN* exon 1. Normal control subjects showed $2^{-(\Delta\Delta CT)}$ values between 0.93 and 1.21. Patients with two copies of *PTEN* displayed values between 0.81 and 1.35, whereas patients with hemizygous deletions (one copy) had values between 0.45 and 0.60.

Table 1

Probes and Primers Used for Real-Time Quantitative Multiplex PCR, Fluorescent Semiquantitative Multiplex PCR Assay, and Promoter Sequencing

Primer/Probe	Sequence (5′→3′)	Amplicon Size (bp)
PTEN E1:		
Forward	GAGGATGGATTCGACTTAGACTTGA	
Reverse	CCCACGTTCTAAGAGAGTGACAGAA	85
Probe	FAM-CCTGTATCCATTTCTG-MGBNF	
PTEN E5:		
Forward	CCTACTTGTTAATTAAAAATTCAAGAGTTTTTT	
Reverse	GTGGGTTATGGTCTTCAAAAGGATA	98
Probe	FAM-TGTGCAACTGTGGTAAA-MGBNF	
RET E8:		
Forward	GTCCTGTGCAGTCAGCAAGAGA	
Reverse	CCACTCACACCTGCCTGTTG	79
Probe	VIC-CCTCACACTCCAGCCG-MGBNF	
PTEN E2:		
Forward	FAM-GTTTGATTGCTGCATATTTCAG	163
Reverse	TGAAATAGAAAATCAAAGCATTC	
PTEN E3:		
Forward	FAM- AAAATCTGTCTTTTGGTTTTTC	178
Reverse	TTGCAAGCATACAAATAAGAA	
PTEN E4:		
Forward	FAM-CATTATAAAGATTCAGGCAAT	205
Reverse	GACAGTAAGATACAGTCTATC	
PTEN E5:		
Forward	FAM-CTTTTTACCACAGTTGCACA	282
Reverse	GGAAAGGAAAAACATCAAAA	
PTEN E6:		
Forward	FAM-CCTGTTAAAGAATCATCTGGA	120
Reverse	AAGGATGAGAATTTCAAGCA	
PTEN E7:		
Forward	FAM- AGGCATTTCCTGTGAAATAA	172
Reverse	TTGATATCACCACACACAGG	
PTEN E8:		
Forward	FAM-CTCAGATTGCCTTATAATAGTC	245
Reverse	TCTGAGGTTTCCTCTGGTC	
PTEN E9:		
Forward	FAM-TCATATTTGTGGGTTTTCATT	260
Reverse	TCATGGTGTTTTATCCCTCT	
RET E8:		
Forward	FAM-CTGTGACCCTGCTTGTCT	135
Reverse	CACTCACACCTGCCTGTT	
Promoter:		
Forward	GCGTGGTCACCTGGTCCTTT	683
Reverse	GCTGCTCACAGGCGCTGA	

PTEN deletions were expected to yield $2^{-(\Delta\Delta CT)}$ values close to 1, whereas samples with hemizygous deletion or duplication were expected to give $2^{-(\Delta\Delta CT)}$ values close to 0.5 or 1.5, respectively. Positive results were controlled on at least three independent experiments. We found three (2.5%) individuals who harbored hemizygous germline deletions encompassing all or part of *PTEN*. The $2^{-(\Delta\Delta CT)}$ values ranged 0.45–0.60, for the three patients with deletions; 0.81–1.35, for the deletion-negative cases; and 0.93–1.21, for the 12 normal individuals tested (fig. 1). One sample (1397-1) showed deletion of all nine *PTEN* exons, which suggests that the deletion encompassed the entire gene. The other two samples (141-2 and 1621-1) had deletions encompassing exons 1–5 and exon 1 only, respectively. No sample was found to have exonic duplication. Further, we included a fragment of *PTEN* exon 5 in the fluorescent multiplex PCR assay and found no deletions except for the ones detected by real-time quantitative multiplex PCR, thereby confirming the sensitivity of fluorescent semiquantitative multiplex PCR assay.

To assess the extent of the *PTEN* germline deletions, three polymorphic markers intragenic to *PTEN* and five polymorphic markers flanking the 10q23.3 region were

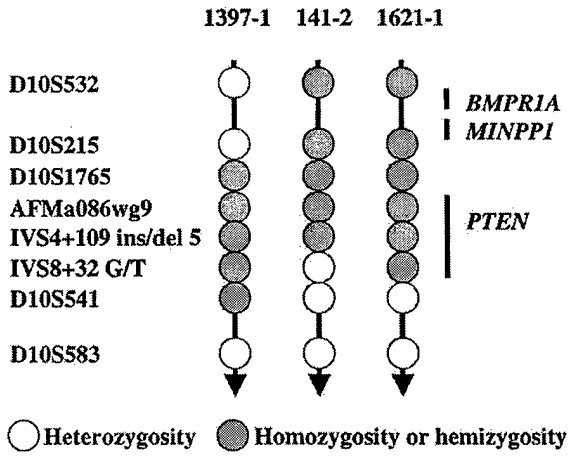

Figure 2 Genotyping results for the three patients with BRRS and/or CS with hemizygous *PTEN* deletions.

genotyped as described elsewhere (Marsh et al. 1999; Zhou et al. 2000*a*) (fig. 2). Markers AFMa086wg9, IVS4+109 ins/del TCTTA (IVS4+109), and IVS8+32 G/T (IVS8+32) are intragenic to *PTEN*, whereas D10S215, D10S1765, and D10S541 are ≤500 Kb up- or downstream of *PTEN*. Markers D10S532 and D10S583 are located ~3 cM upstream and 5 cM downstream of *PTEN*, respectively. All three patients with deletions were apparently homozygous (which suggests hemizygosity) at markers closest to or within the *PTEN* gene (D10S1765, AFMa086wg9, IVS4+109, and IVS8+32), consistent with the hemizygous deletions already identified by real-time quantitative multiplex PCR and fluorescent multiplex PCR assays. In one case (1397-1), "homozygosity" spanned from the upstream marker D10S1765 to D10S541, downstream of *PTEN*, concordant with whole-gene deletion detected by the quantitative PCR assays, and suggesting the deletion which also includes all of the *PTEN* promoter and likely all of the 3′ UTR. The second deletion case (141-2) showed "homozygosity" at D10S215, D10S1765, AFMa086wg9, and IVS4+109 and heterozygosity at IVS8+32, consistent with partial *PTEN* deletion, encompassing exons 1–5. In the third case (1621-1), although the homozygosity spanned from D10S215 to IVS8+32, deletion was detected only at exon 1 by real-time quantitative PCR assay, which suggests that the three intragenic markers are truly homozygous. All these deletions likely extend to at least 50 kb upstream of the translational start site (D10S1765). To investigate whether these three deletions include another gene close to the 10q23.3 region, we analyzed *MINPP1*, located ~500 kb upstream of *PTEN*, using semiquantitative duplex PCR assay; no evidence of involvement of this gene was found, thus also excluding the relevant juvenile polyposis gene *BMPR1A* (upstream of *MINPP1*) as part of the deletion (fig. 2).

Germline DNA from the remaining 119 patients without deletions was subsequently subjected to sequence analysis for mutations in the 600-bp full-promoter region of *PTEN*. Primers were designed to amplify the full promoter region between −1344 bp and −745 bp upstream of the translation start codon (Sheng et al. 2002) (table 1). Ten heterozygous sequence variants within the *PTEN* promoter region were found in nine patients with CS (9/119, 7.6% of total; 9/97, 10% of CS) (fig. 3). None of these promoter sequence variants were found among

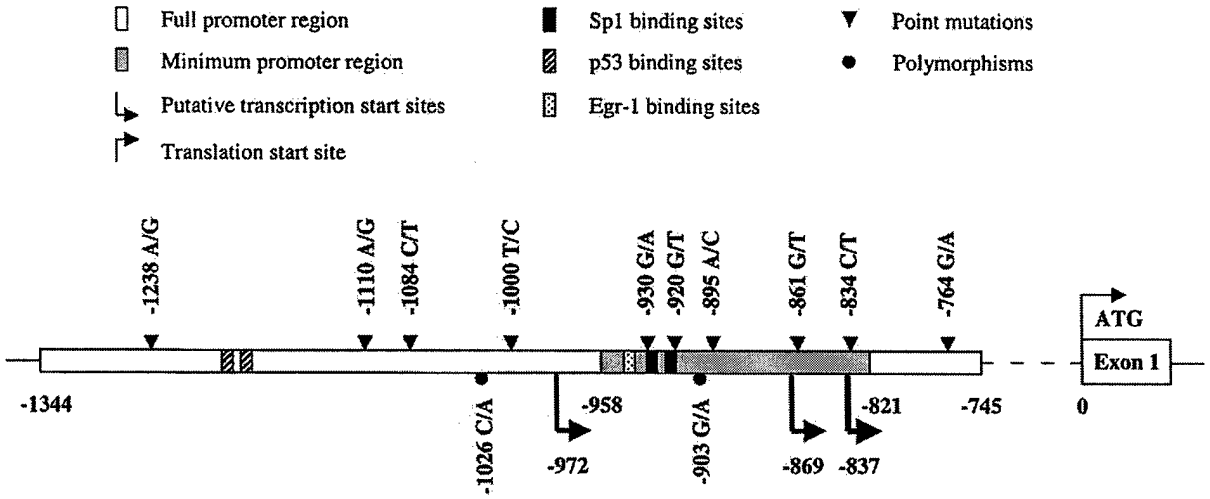

Figure 3 Germline *PTEN* promoter mutations and polymorphisms found in probands with CS

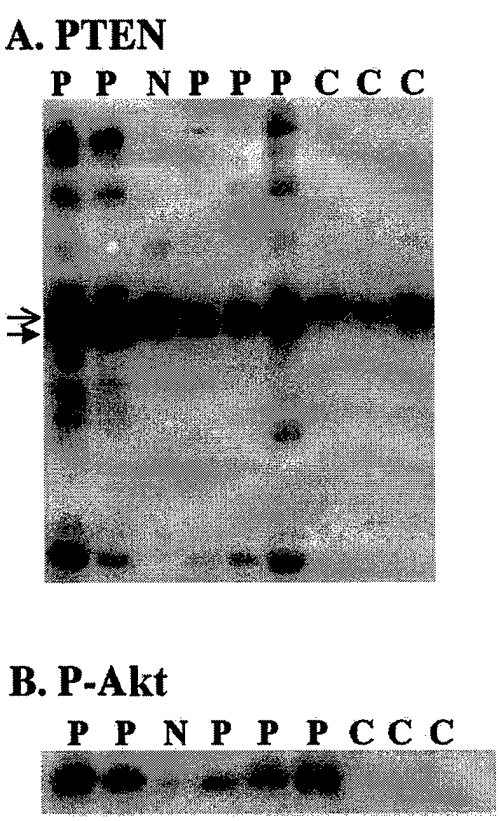

Figure 4 Aberrant PTEN protein species and increased phosphorylation of Akt in promoter-mutation samples. Samples from patients with promoter mutations (P), a patient who is *PTEN*-mutation negative (N), and normal control subjects (C) were analyzed. *A*, Western analysis for PTEN protein. Open arrows indicate the expected molecular weight of PTEN; closed arrows indicate the slower migrating band. *B*, Western analysis for P-Akt.

186 normal white control subjects (372 chromosomes), which suggests that the former are likely pathogenic. Two other sequence variants (−903 G/A and −1026 C/A) were present in both patients and normal control individuals with similar allele frequencies (data not shown), which suggests that they were indeed polymorphisms.

To functionally assess the *PTEN*-promoter-point mutations, cellular protein from five case subjects with promoter mutations was obtained during Trizol isolation of RNA and was subjected to western blot analysis (Waite and Eng 2003). Control samples and a sample from a patient with CS who was negative for the *PTEN* mutation display a single immunoreactive protein of the correct size (fig. 4). It is interesting that samples from patients carrying the promoter mutations showed a decrease in PTEN protein of the correct molecular weight (fig. 4*A*, *open arrowhead*), concordant with a dramatic increase of a slightly lower band (fig. 4*A*, *closed arrowhead*). This lower band is visible in the *PTEN*-mutation–negative CS sample and in an occasional control sample but not to the same extent as the promoter-mutation samples and never with a loss of the correct PTEN band. Three of the five promoter-mutation–positive cases had a laddering effect, with several bands recognized at both lower and higher molecular weights (fig. 4*A*), which has not been observed in 32 control samples or 23 *PTEN*-mutation–negative samples (data not shown). These data strongly suggest that the lower molecular weight band and the laddering effect are specific and related to the promoter mutations in these patients.

We next investigated if the PTEN protein produced in these patients was active. PTEN antagonizes the PI3K/Akt pathway by decreasing phosphatidylinositol-3,4,5 triphosphate levels, which results in decreased Akt phosphorylation (reviewed by Waite and Eng [2002]). Thus, active PTEN results in low Akt phosphorylation, and deficient PTEN results in increased Akt phosphorylation. Figure 4*B* shows that, in control and *PTEN*-mutation–negative samples, the level of phosphorylated Akt (detected by an antiphospho-Akt antibody) is low to undetectable, which indicates active PTEN function. In contrast, the level of phosphorylated Akt in the samples from cases carrying promoter mutations are dramatically elevated (fig. 4*B*). These data indicate that the PTEN protein produced has nonfunctional lipid phosphatase activity.

It is interesting to note that PTEN is a dual-substrate phosphatase that dephosphorylates both lipid and protein substrates (reviewed by Waite and Eng [2002]). At this time, we can accurately assess only the lipid phosphatase activity by monitoring the levels of Akt phosphorylation. Although our lab has shown that the protein phosphatase activity of PTEN regulates the down regulation of the mitogen-activated protein kinase pathway (Weng et al. 2001), we have found that the level of activation of this pathway varies considerably, even in normal controls (K. A. Waite and C. Eng, unpublished observations). Therefore, we are unable to reliably investigate changes in the protein phosphatase activity of PTEN that may arise from various *PTEN* mutations. It is interesting to postulate that various degrees of changes in both the lipid and protein phosphatase activities may play a role in the wide range of clinical manifestations of CS and BRRS.

There is little doubt that all three deletions are functionally deleterious, as all three likely include the promoter as well as all or part of *PTEN*. Protein analysis on the lymphoblastoid cell lines of one of the three deletion-positive patients revealed ~50% reduction in PTEN protein level, consistent with hemizygosity of *PTEN* (K. A. Waite and C. Eng, unpublished data). All three patients had a diagnosis of BRRS or BRRS/CS overlap. Two of these probands have gastrointestinal polyposis. Although our conclusion is based on a small sample size, there is

a trend toward gastrointestinal hamartomatous polyposis in individuals with deletions, compared with the 119 patients with CS or BRRS without deletions ($P = .1$; Fisher's two-tailed exact test). Although our patients' deletions are not cytogenetically obvious, at least three other unrelated patients with CS or BRRS have been reported elsewhere to have deletions or rearrangements in the *PTEN* region detected by cytogenetics (Arch et al. 1997; Tsuchiya et al. 1998; Marsh et al. 1999; Ahmed et al. 2000). All three with cytogenetically detected *PTEN* deletion or rearrangements carried the clinical diagnosis of BRRS. Over all, therefore, at least five probands with BRRS have been found to have deletions of or encompassing *PTEN*. Given that hemizygous *PTEN* deletions detected in probands with BRRS encompassed from single exon to whole gene, it is very likely that certain "neat" single-exon deletions—such as the one in patient 1621–1, involving only exon 1—could be missed by use of FISH technique with application of a designed probe.

Until now, the *PTEN* promoter had not been examined in patients with CS and/or BRRS. However, in vitro work has shown that activated PPARγ and p53 result in up-regulated PTEN transcription (Patel et al. 2001; Stambolic et al. 2001; Virolle et al. 2001), which suggests that alterations of the promoter sequence could result in changes to PTEN protein structure, levels, and function. Among 119 mutation-negative or deletion-negative CS or BRRS cases, nine probands with CS were found to carry germline heterozygous point mutations in the promoter. Of significance, all nine individuals with *PTEN*-promoter mutations had a diagnosis of classic CS yet had relatively mild phenotypic features, as operationally defined by oligo-organ involvement (involvement of fewer than four organs; see Marsh et al. [1998] for classification) (table 2). It is interesting that one deletion-positive proband with exon 1 and upstream involvement had similarly mild features, with only two-organ involvement, macrocephaly, and lipomas. The deletion encompassing exons 1–5 in patient 141-2 was also present in a sibling, 141–1, both of whom have features of both CS and BRRS. It would be interesting to see whether these promoter mutations are also present in affected relatives of familial cases. Collection and analysis of parental DNA of these case subjects with promoter mutations are ongoing.

Of the 10 promoter mutations (one patient had two different sequence variants), 5 were localized to the minimum *PTEN* promoter region (−958 to −821), 2 of which (−920G→T and −930G→A) are predicted to alter two putative Sp1 transcription factor–binding sites (fig. 3). Further, protein analysis revealed a reduced expression of wild-type PTEN, a strong lower–molecular-weight immunoreactive band, and a laddering effect of protein immunoreactive with a specific monoclonal antibody against human PTEN, which suggests that these point nucleotide substitutions are functionally significant and, thus, represent promoter mutations. These data suggest that the promoter variants may result in alternative start sites that yield PTEN protein of various sizes (fig. 4*A*).

It is interesting to note that the two samples with mutations at the two putative Sp1 binding sites were the two with doublet PTEN-immunoreactive bands but no laddering effect (fig. 4*A*). Although the transcriptional regulation of *PTEN* is only now beginning to be elucidated, we suspect that these variants would alter *PTEN* transcription, which would result in impaired protein expression. The presence of some wild-type protein, together with PTEN proteins of various sizes, might be postulated to result in the milder phenotype associated with these promoter variants. It is also possible that such mutations result in posttranslational modifications, which could result in altered mobility during SDS-PAGE. Another possibility is that the PTEN protein formed is altered at the protein level, which results in targeted degradation, and it is the degradation of PTEN protein that we are observing. The mechanisms of PTEN degradation are only now being understood (Vazquez et al. 2000; Torres and Pulido 2001; Waite and Eng 2003). Improper PTEN degradation could also result in impaired protein expression. Further analysis, as patient sample material

Table 2

Family-as-Unit Clinical Features of Probands Positive for *PTEN* Promoter Mutation

Multiorgan[a]	Breast Cancer	Thyroid Cancer	Uterine Cancer	Mutation
No	No	No	No	−1000TC, −1238A→G
No	Yes	No	No	−1110A→G
No	No	No	No	−1084C→T
No	No	Yes	No	−930G→A
No	Yes	No	No	−920G→T
No	Yes	No	No	−895A→G
Yes	Yes	Yes	No	−861G→T
No	Yes	Yes	No	−834C→T
No	Yes	No	No	−764A→G

[a] Multiorgan involvement operationally defined as at least five organs involved, as detailed by Marsh et al. (1998).

becomes available, will be necessary to determine the exact mechanism of the laddering effect.

Regardless of the mechanism of lower molecular weight proteins, we have demonstrated that the PTEN protein species produced in these promoter-mutation–positive patients is deficient. The levels of phosphorylated-Akt were significantly higher in samples haboring promoter sequence variants, compared with control subjects and *PTEN*-mutation–negative samples (fig. 4*B*), which indicates an increase in the activity of the pro-proliferative PI3K/Akt pathway.

On the basis of our observations that 11% of individuals with features of BRRS were found to have a deletion, it may be prudent to offer deletion analysis to patients with BRRS and patients with CS/BRRS with gastrointestinal polyposis but without PCR-based intragenic *PTEN* mutations. Further, our ~10% promoter-mutation frequency among probands with CS previously found to be PCR-mutation negative and deletion negative does suggest that promoter analysis might be useful in the clinical setting. That these promoter variants are deleterious and likely causative of CS has been demonstrated by aberrant PTEN protein bands on western blot, which resulted in activation of the pro-proliferative Akt pathway. Indeed, western analyses should be considered a useful molecular diagnostic adjunct to determine functionality of promoter variants.

Acknowledgments

We are grateful to our patients and families and their clinicians, who have continued to participate in our research. We thank Susan Whitman and Tamara Vukosavljevic (Real-time PCR Core Facility at the Comprehensive Cancer Center, The Ohio State University), for their excellent technical support; and Micheala Aldred, for donating anonymous DNA samples from normal control subjects. This work is partially funded by the American Cancer Society (RSG-02-151-01-CCE to C.E.), Susan G. Komen Breast Cancer Research Foundation (BCTR 2000 462 to C.E.), and National Cancer Institute (P30CA16058 to The Ohio State University Comprehensive Cancer Center). C.E. is a recipient of the Doris Duke Distinguished Clinical Scientist Award.

Electronic-Database Information

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CS, BRRS, PS, and *PTEN/MMAC1/TEP1*)

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